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(54) Title: BRAIN DERIVED NEUROTROPHIC FACTOR

(57) Abstract

The present invention relates to nucleic acid sequences encoding brain derived neurotrophic factor (BDNF), as well as BDNF protein produced in quantity using these nucleic acid sequences, as well as fragments and derivatives thereof. In addition, the invention relates to pharmacologic compositions and therapeutic uses of BDNF, having provided, for the first time, the means to generate sufficient quantities of substantially pure BDNF for clinical use. In a specific embodiment, BDNF may be used to promote the survival of substantia nigra dopaminergic neurons and basal forebrain cholinergic neurons, thereby providing a method for treating, respectively, Parkinson's disease and Alzheimer's disease. The invention also relates to antibodies directed toward BDNF or fragments thereof, having provided a method for generating sufficient immunogen. Further, by permitting a comparison of the nucleic acid sequences of BDNF and NGF, the present invention provides for the identification of homologous regions of nucleic acid sequence between BDNF and NGF, thereby defining a BDNF/NGF gene family; the invention provides a method for identifying and isolating additional members of this gene family.

DESIGNATIONS OF "DE"

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BRAIN DERIVED NEUROTROPHIC FACTOR

1.   INTRODUCTION

The present invention relates to nucleic acid sequences encoding brain derived neurotrophic factor (BDNF), to the substantially pure protein, peptide fragments or derivatives produced in quantity therefrom, and to antibodies directed toward BDNF protein, peptide fragments, or derivatives. In addition, the invention relates to genes that are members of a newly defined BDNF/NGF gene family, and to their gene products. The invention also relates to pharmaceutical compositions comprising effective amounts of BDNF gene products or, alternatively, antibodies directed toward BDNF gene products, and to methods of diagnosing and treating a variety of neurological diseases and disorders, including Alzheimer's disease and Parkinson's disease. In particular, the BDNF gene products of the invention have value in the diagnosis and therapy of disorders of dopaminergic neurons, such as Parkinson's disease, as well as disorders of sensory neurons and degenerative diseases of the retina.

2.   BACKGROUND OF THE INVENTION

2.1.   NEURONAL CELL DEATH AND THE ROLE OF NEUROTROPHIC FACTORS IN THE DEVELOPMENT OF THE NERVOUS SYSTEM

Throughout many parts of the vertebrate nervous system many more neurons are present during early development than are found in the adult animal. Periods of early development are characterized by waves of naturally occurring neuronal cell death (Carr and Simpson, 1978, J. Comp. Neurol., 182:727-740; Cowan et al., 1984, Science 225:1258-1265). The survival, differentiation and maturation of developing neurons may be regulated by environmental or 'epigenetic' factors rather than by a strict intrinsic genetic program. For example, experimental

manipulations of the chick embryo have shown that
transplantation or extirpation of peripheral "target
fields", such as a limb bud or the eye, at early stages in
chick development can result in a corresponding increase or
decrease, respectively, in the number of sensory,

5   sympathetic, parasympathetic or motorneurons adjacent to the
enlarged or depleted target field (Hamburger, 1934, J. Exp.
Zool. 68:449; Hollyday and Hamburger, 1976, J. Comp.
Neurol., 170:311-321; Landmesser and Pilar, 1976, J. Cell.
Biol., 68:357-374). A target field may only support a

10 limited number of neurons, and a normal part of the
developmental process may be the pruning of an excess number
of neurons to match the "neurotrophic" capacity of the
target tissue. The discovery and isolation of the protein
now called nerve growth factor (NGF) has led to a molecular

15 hypothesis of how a target may be able to regulate the
number of neurons which survive and innervate that tissue
(Levi-Montalcini et al., 1968, Physiol Rev., 48:524-569;
Thoenen and Barde, 1980, Physiol Rev., 60:1284-1335).

It is now well established, at least in the

20 peripheral nervous system, that neuronal target tissues
synthesize and release limited quantities of various
neurotrophic molecules which are critical to the survival of
specific types of neurons (Korsching and Thoenen, 1983,
Proc. Natl. Acad. Sci. U.S.A. 80:3513-3516; Heumann et al.,

25 1984, EMBO J. 3:3183-3189; Shelton and Reichardt, 1984,
Proc. Natl. Acad. Sci U.S.A. 81:7051-7955; Korsching and
Thoenen, 1985, Neurosci. Lett. 54:201-205).

2.2.   NERVE GROWTH FACTOR

30   Nerve growth factor (NGF) is by far the most fully
characterized of these neurotrophic molecules and has been
shown, both in vitro and in vivo, to be essential for the
survival of sympathetic and neural crest-derived sensory

35 neurons during early development of both chick and rat

(Levi-Montalcini and Angeletti, 1963, Develop. Biol. 7:653-659; Levi-Montalcini et al., 1968, Physiol. Rev. 48:524-569). Injections of purified NGF into the developing chick embryo have been found to cause massive hyperplasia and hypertrophy of spinal sensory neurons and sympathetic neurons (Levi-Montalcini and Booker, 1960, Proc. Natl. Acad. Sci. U.S.A. 46:373-384; Hamburger et al., 1981, J. Neurosci. 1:60-71). Conversely, removal or sequestration of endogenous NGF by daily injection of anti-NGF antibodies into neonatal rats has been associated with virtual destruction of the sympathetic nervous system (Levi-Montalcini and Booker, 1960, Proc. Natl. Acad. Sci. U.S.A. 46:384-391; Levi-Montalcini and Angeletti, 1966, Pharmacol. Rev. 18:619-628). Exposure to NGF antibodies even earlier in development either by antibody injections in utero or by passive transplacental transfer of maternal antibodies has been shown to result in a substantial loss of neural crest-derived sensory neurons such as spinal and dorsomedial trigeminal sensory neurons (Goedert et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:1580-1584; Gorin and Johnson, 1979, Proc. Natl. Acad. Sci. U.S.A. 76:5382-5386). Until recently, almost all studies of NGF had focused on its role in the peripheral nervous system, but it now appears that NGF also influences the development and maintenance of specific populations of neurons in the central nervous system (Thoenen et al., 1987, Rev. Physiol. Biochem. Pharmacol. 109:145-178; Whittemore and Seiger, 1987, Brain Res. Rev. 12:439-464).

The serendipitous discovery of large amounts of NGF protein in the submandibular gland of the adult male mouse (Cohen, 1960, Proc. Natl. Acad. Sci. U.S.A. 46:302-311) and an earlier discovery of high levels of NGF in snake venom (Cohen and Levi-Montalcini, 1956, Proc. Natl. Acad. Sci. U.S.A. 42:571-574) fortuitously provided sufficient quantities of NGF to permit studies relating to the

physiology, protein chemistry, and, more recently, the
molecular biology of NGF (i.e. molecular cloning of NGF and
its receptor). The function of large amounts of NGF in the
adult male mouse salivary gland remains unknown; however, it
appears that this abundant source of NGF may not play any
5   role in development or maintenance of the peripheral or
central nervous system.   In target tissues innervated by
NGF sensitive neurons (neurons which have been shown to
depend on NGF for survival, to possess high affinity NGF
receptors and to internalize and retrogradely transport NGF
10 with high specificity), the steady state of measurable
levels of NGF have been found to be extremely low, in the
range of picograms or nanograms per gram of tissue, compared
to thousand-fold higher levels in adult male mouse salivary
gland tissue. NGF has not been found at any appreciable
15 level in serum and therefore does not appear to be a
circulating growth factor or hormone (Suda et al., 1978,
Proc. Natl. Acad. Sci. U.S.A. 75:4042-4046).

        In addition to the important discovery of a major
source of NGF protein in the mouse submandibular gland, the
20 development of sensitive, reliable and efficient biological
assays has played a major role in elucidating the biology
and biochemistry of NGF. Dorsal root ganglia (DRG) of the
developing chick embryo were one of the first neuronal types
to be shown to be responsive to NGF in vitro. Explant
25 cultures of E8-E12 chick DRG in a plasma clot, and, more
recently, dissociated neuron-enriched cultures of chick DRG
have proven very useful in bioassays for NGF activity (e.g.
during purification) and for in vitro studies of NGF biology
(Levi-Montalcini et al., 1954, Cancer Res. 4:49-57; Levi-
30 Montalcini and Angeletti, 1963, Develop. Biol. 1:653-659;
Greene, 1977, Develop. Biol. 58:96, ibid 58:106). The fact
that more than forty DRG can be dissected from a single
chick embryo has led to the widespread use of NGF bioassays
in many laboratories.
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In addition to the availability of large
quantities of NGF protein and efficient NGF assay systems, a
third major factor which has greatly contributed to our
understanding of the biology of NGF is the relative ease
with which antibodies to NGF can be raised in guinea pigs,

5   rabbits, sheep, etc.; it appears that mouse NGF is highly
immunogenic. Cohen (1960, Proc. Natl. Acad. Sci. U.S.A.
46:302-311) raised antibodies to the NGF he had purified
from mouse submandibular gland and showed, with Levi-
Montalcini and Booker (1960, Proc. Natl. Acad. Sci. U.S.A.

10   46:384-391) that these antibodies caused destruction of
sympathetic ganglia, or "immunosympathectomy," when
administered daily to newborn rats (Levi-Montalcini and
Angeletti, 1966, Pharmacol. Rev. 18:619-628).

        The abundance of NGF protein allowed the primary

15   sequence to be determined by relatively conventional protein
chemistry (Angeletti and Bradshaw, 1971, Proc. Natl. Acad.
Sci. 68:2417-2420). The NGF gene has now been cloned from
many species, including mouse (Scott et al., 1983, Nature
302:538-540), human (Ullrich et al., 1983, Nature 303:821-

20   825), cow and chick (Meier et al., 1986, EMBO J. 5:1489-
1493), and rat (Whittemore et al., 1988, J. Neurosci. Res.,
20:402-410) using essentially conventional molecular biology
based on the availability of the protein sequence of mouse
NGF to design suitable oligonucleotide probes. The

25   availability of abundant NGF has also greatly facilitated
studies on the NGF receptor, which have ultimately led to
the molecular cloning of the NGF receptor from human and rat
(Johnson et al., 1986, Cell, 47:545-554; Radeke et al.,
1987, Nature 325:593-597).

30   It is now well established that NGF is not a
ubiquitous neurotrophic factor. Within the peripheral
nervous system, NGF appears not to be a survival factor for
parasympathetic neurons, neural placode-derived sensory

35   neurons or enteric neurons, as determined both from studies

in _vitro_ and _in vivo_. Furthermore, NGF does not appear to
be a survival factor for developing motorneurons (Oppenheim,
1982, J. Comp. Neurol. 210:174-189), although these neurons
do appear to express at least a low affinity form of the NGF
receptor during development (Raivich et al., 1985, EMBO J.
4:637-644). The lack of effects of NGF on these neuronal
types has prompted the search for other neurotrophic
factors, especially factors that would sustain the survival
of spinal cord motorneurons and/or parasympathetic neurons
of the ciliary ganglion.

2.3. OTHER NEUROTROPHIC FACTORS

In the past decade there have been numerous
reports of neurotrophic activity in extracts of a great
variety of tissues and in the conditioned culture medium of
many different cell types. In almost all cases, however,
progress in purifying and characterizing these activities
has been hampered by the fact that such activities are
present in extremely small amounts, in the range of
picograms to nanograms per gram of tissue.

Furthermore, whereas adequate bioassays have been
established for peripheral neurons, designing reliable,
reproducible and specific assays for central nervous system
neurons has proved problematic. While individual types of
peripheral neurons are found as discrete, easily dissectable
ganglia, central nervous system (CNS) neurons are invariably
highly heterogenous in their distribution. Thus, specific
markers are required for either identification or enrichment
of particular classes of CNS neurons. Progress in producing
such markers, for example, antibodies directed toward cell
surface or cytoskeletal components, or specific histological
stains, has been very limited. Accordingly,
characterization of neurotrophic factors which are (i) not
as abundant as NGF, (ii) difficult to assay, and (iii) not
available in sufficient quantities to elicit antibody

production, has proved to be an exceedingly difficult
process.

2.3.1. COMPARISON OF A BRAIN DERIVED NEUROTROPHIC FACTOR (BDNF) AND NERVE GROWTH FACTOR

5   Neurotrophic activity capable of sustaining the
survival of embryonic chick dorsal root ganglion neurons in
vitro was identified in the "conditioned medium" in which
rat C-6 glioma cells had been cultured (Barde et al., 1978,
Nature 274:818). The activity was not neutralized by
10 antibodies to mouse NGF, suggesting the presence of another
neurotrophic factor in the conditioned medium. Similar
activities that could not be blocked by NGF antibodies were
subsequently reported in cultures of normal adult rat brain
astroglial cells (Lindsay, 1979, Nature 282:80-82; Lindsay
15 et al., 1982, Brain Res. 243:329-343) and in extracts of
developing and adult rat brain (Barde et al., 1980, Proc.
Natl. Acad. Sci. U.S.A. 77:1199-1203) and developing and
mature chick spinal cord (Lindsay and Peters, 1984,
Neurosci. 12:45-51). However, in no case was the active
20 factor(s) isolated or identified, and it remains
questionable as to whether the observed activities were due
to the same or different factor(s).

        Using pig brain as a starting material, Barde et
al. (1982, EMBO J. 1:549-553) reported a factor, now termed
25 brain-derived neurotrophic factor (BDNF), which appeared to
promote the survival of dorsal root ganglion neurons from
E10/E11 chick embryos. The neurotrophic activity was found
to reside in a highly basic protein (isoelectric point, pI >
10.1) which migrated during sodium dodecyl sulfate (SDS)
30 gel electrophoresis as a single band of 12.3 kD molecular
weight. The purification factor was estimated to be 1.4 x
10^6, but the yield was very low, with only approximately 1
μg of BDNF purified from 1.5 kg of pig brain. Furthermore,
because the last step in the purification process was
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preparative gel electrophoresis, the activity of BDNF could not be fully renatured secondary to the presence of residual SDS (Barde and Thoenen, 1985, in "Hormones and Cell Regulation," Vol. 9, Dumont et al., eds. Elsevier Science Publishers, pp. 385-390). It was noted that the highly basic nature and molecular size of BDNF were very similar to the NGF monomer. However, BDNF appeared to have properties that differed from the known properties of NGF in that (a) in the chick dorsal root ganglion bioassay, antibodies to NGF had no apparent effect on the biological activity of BDNF; (b) the effects of BDNF and NGF appeared to be additive; and (c) unlike NGF, BDNF was found to have no effect on the survival of E12 chick sympathetic neurons. In addition, during early studies with brain extracts, it was observed that the neurotrophic activity in these sources appeared to act upon sensory neurons at later stages of development than were associated with NGF. Using dissociated cultures of chick embryo neurons cultured on a polycationic substrate such as polylysine or polyornithine, BDNF was found to support the survival of more than 30 per cent of E10-11 (embryonic day ten or eleven) dorsal root ganglion neurons but seemed to have little effect on the survival of the same neurons at E6 (Barde et al., 1980, Proc. Natl. Acad. U.S.A. 77:1199-1203 supra). Under similar conditions, NGF supported the survival of 30-40 percent of E6 DRG neurons. Interestingly, it was later found that when cultured on a substrate coated with the extracellular matrix glycoprotein laminin, both NGF and BDNF supported the survival of about 50 per cent of DRG neurons from chick embryos of ages E6-E12 (Lindsay et al., 1985, Develop. Biol. 112:319-328). In the latter study, the effects of NGF and BDNF were found to be additive when both were present at saturating concentrations.

Early studies by Levi-Montalcini (1966, The Harvey Lectures 60:217-259) on the neuronal specificity of NGF

suggested that NGF was not a ubiquitous neurotrophic factor
even for sensory neurons, as NGF appeared to have no effect
upon neurons of certain cranial sensory ganglia of the
chick, especially the nodose ganglion of the tenth cranial
nerve.   Later in vivo studies (Johnson et al., 1980, Science

5   210:916-918; Pearson et al., 1983 Develop. Biol. 96:32-36)
showed that NGF deprivation during embryogenesis had no
effect on the survival of neurons in most cranial sensory
ganglia of the rat, while similar treatment greatly depleted
the neuronal count in sensory ganglia derived from the

10 neural crest.   More detailed in vitro studies (Lindsay and
Rohrer, 1985, Develop. Biol. 112:30-48; Davies and Lindsay,
1985, Develop. Biol. 111:62-72; Lindsay et al., 1985, J.
Cell. Sci. Suppl. 3:115-129) clearly indicated that NGF
supports the survival of most neural crest-derived sensory

15 neurons but has no apparent effect on the survival of
cranial sensory neurons derived from neural placodes.

        The first demonstration of a neuronal specificity
of BDNF distinct from that of NGF was the demonstration in
vitro that purified BDNF supports the survival of 40-50% of

20 sensory neurons dissociated from the neural placode-derived
nodose ganglion of the chick embryo at E6, E9 or E12
(Lindsay et al., 1985, J. Cell. Sci. Supp. 3:115-129). NGF
was without apparent effect on these neurons either by
itself or in conjunction with BDNF. It was later shown in

25 explant culture studies that BDNF appeared to support
survival and neurite outgrowth from other neural placode-
derived sensory ganglia, including the petrosal, geniculate
and ventrolateral trigeminal ganglia (Davies et al., 1986,
J. Neurosci. 6:1897-1904), none of which have been found to

30 be sensitive to NGF. In all of the above studies antibodies
to NGF had no effect upon the observed activity of BDNF. In
addition to its effects on cultured neurons from peripheral
ganglia, BDNF was found to stimulate survival and neuronal

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differentiation of cells cultured from quail neural crest (Kalcheim and Gendreau, 1988, Develop. Brain Res. 41:79-86).

Prior to the instant invention, the inability to produce sufficient amounts of BDNF for immunization prevented the production of anti-BDNF antibodies for

5 comparison to anti-NGF antibodies in their effects on neuronal populations, and precluded BDNF/NGF cross-neutralization experiments. Two recent studies with BDNF (Kalcheim et al., 1987, EMBO J. 6:2871-2873; Hofer and Barde, 1988, Nature 331:261-262) have, however, indicated a

10 physiological role of BDNF in avian PNS development. If a mechanical barrier was placed in ovo between E3/E4 DRG (embryonic day 3 or 4 dorsal root ganglia) and their CNS target in the neural tube, many DRG neurons were observed to die (Kalcheim and Le Douarin, 1986, Develop. Biol. 116:451-

15 466). It was postulated that this neuronal death may have been due to deprivation from a CNS (neural tube) derived neurotrophic factor. It was subsequently observed that BDNF attached to a laminin-coated sialastic membrane could prevent this cell death (Kalcheim et al., 1987, EMBO J.

20 6:2871-2873). Injections of BDNF into developing quail eggs has been found to reduce naturally occurring cell death in the nodose ganglia, an effect not seen with NGF (Hofer and Barde, 1988, Nature 331:261-262). In addition to its effect on peripheral sensory neurons of both neural crest and

25 neural placode origin, BDNF was found to support the survival of developing CNS neurons. Johnson et al. (1986, J. Neurosci. 6:3031-3938) presents data that indicates that BDNF supports the survival of retinal ganglion cells cultured from E17 rat embryos. This was in agreement with

30 previous studies which showed that conditioned media and brain extracts prepared from the target regions of retinal ganglion cells appeared to support the survival of these neurons (McCaffery et al., 1982, Ex. Brain Res. 48:37-386;

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Sarthy et al., 1983, J. Neurosci. 3:2532-2544; Turner et
al., 1983, Dev. Brain Res. 6:77-83).

In addition to its effects on the survival of
developing neurons in culture, BDNF has been shown to have
effects on cultured adult peripheral and central nervous
5   system neurons. BDNF, as well as NGF, has been shown to
stimulate axonal regeneration from adult rat DRG neurons in
culture (Lindsay, 1988, J. Neurosci. 8:2394-2405) although
adult sensory neurons did not appear to require neurotrophic
factors for maintenance in vitro over 3 or 4 weeks.
10 Furthermore, in cultures of adult rat retina, BDNF was
observed to promote both survival and axonal elongation from
retinal ganglion cells (Thanos et al., 1989, Eur. J.
Neurosci. 1:19-26). A comparison of the biological effects
of NGF and BDNF is presented in Table I.

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TABLE I

COMPARISON OF BIOLOGICAL ACTIVITIES OF
BDNF AND NGF*

			SURVIVAL**	
PERIPHERAL NERVOUS SYSTEM			BDNF	NGF
(i)	E6	Chick DRG	-	++
	E10	Chick DRG	-	++
	E12	Chick Sympathetic neurons	-	++
(ii)	E6 - E12	Chick DRG	++	++
	E6 - E12	Chick Nodose	++	-
	E12	Chick Sympathetic neurons	-	++
	E12	Chick ciliary	-	-
	(Lindsay et al., 1985, supra)			
(iii)	E3 - E14	Chick:		
		Jugular	+/++	++
		DM-trigeminal	+/++	++
		Petrosal	+/++	-
		Geniculate	+/++	-
		VL-trigeminal	++	-
		Vestibular	-	-
		Mesencephalic	++	-
	(Davies et al., 1986, supra)			
	(Barde et al., 1987, Prog. Brain Res., 71:185-189)			
CENTRAL NERVOUS SYSTEM				
(i)	E17 Rat Retinal Ganglion Cells		++	-
	(Johnson et al., 1986, J. Neurosci. 63031-3038)			

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*   in chronological order according to publication date; effects tested in vitro

**   no survival:(-); moderate survival (+); good survival (++)

2.3.2.   NEURONAL TARGETS OF BRAIN DERIVED NEUROTROPHIC FACTOR

Sensory neurons of peripheral nerve ganglia have been found to arise from either of two distinct, transient

embryological structures, namely, the neural crest and neural
placodes. The neural crest appears to give rise to both
neurons and satellite cells of autonomic ganglia and spinal
nerve sensory ganglia, i.e. DRG. The contribution of the
neural crest and neural placodes to the formation of cranial

5 nerve sensory ganglia has been studied using the quail/chick
chimera transplantation system devised by Le Douarin (Le
Douarin, 1973, Develop. Biol. 20:217-222; Noden, 1978,
Develop. Biol. 67:313-329; Narayanan and Narayanan, 1980,
Anat. Rec. 196:71-82; Ayer-Le Lievre and Le Douarin, 1982,

10 Develop. Biol. 94:291-310; D'Amico-Maratel and Nodem, 1983,
Am. J. Anat. 166:445-468). As reviewed in Lindsay et al.
(1985, J. Cell. Sci. Supp. 3:115-129), it is now believed,
at least for birds, that neurons of the distal ganglia of
the VIIth, IXth, and Xth cranial nerves (geniculate,

15 petrosal and nodose ganglia, respectively) and neurons of
the vestibuloacoustic complex of the VIIIth cranial nerve
are exclusively of neural placode origin. The trigeminal
ganglion of the Vth cranial nerve contains neurons of both
crest and placode origin (with the placode-derived neurons

20 predominant in the ventrolateral pole of the maxillo-
mandibular lobe) whereas the satellite cells of all cranial
ganglia have been found to be entirely of neural crest
origin.

From in vitro experiments using both explant and

25 dissociated, neuron-enriched cultures of spinal and cranial
nerve sensory neurons, it has been found that sensory
neurons of neural crest origin are responsive to NGF; in
contrast, neurons derived from neural placodes (including
neurons of the ventrolateral portion of the trigeminal

30 ganglion and the entire neuronal population of the
vestibular, geniculate, petrosal and nodose ganglia) have
been observed to be largely unresponsive to NGF throughout
embryonic development. In contrast to differences in their

35 requirement and responsiveness to NGF, both placode and

neural crest derived sensory neurons have been found (Table
I) to be responsive to the survival and neurite-promoting
activity of BDNF (Lindsay et al., 1985, J. Cell. Sci. Supp.
$\underline{3}$:115-129; Lindsay et al., 1985, Develop. Biol. $\underline{112}$:319-328
Kalcheim and Gendreau, 1988, Develop. Brain Res. $\underline{41}$:79-86).

5   Tebar and Barde (1988, J. Neurosci. $\underline{8}$:3337-3342) studied the
binding parameters of radioactively labeled BDNF to chick
embryo dorsal root ganglion neurons; their results are
consistent with the existence of two classes of BDNF
receptors, one with high affinity for BDNF, the other with

10 low affinity. No high affinity receptors were observed on
sympathetic neurons.

        The known neuronal targets of BDNF were further
reviewed by Barde et al. (1987, Prog. Brain Res. $\underline{71}$:185-
189). Prior to the instant invention, identification of

15 cells synthesizing BDNF has not been feasible due to the
lack of nucleic acid or antibody probes specific for BDNF.
Attempts to prepare either polyclonal or monoclonal
antibodies to BDNF have been unsuccessful. This failure to
raise antibodies had hindered the molecular cloning of BDNF,

20 determination of the physiological effect of depriving
developing neurons of BDNF in vivo, quantitation of BDNF in
tissues using an immunoassay, and localization of BDNF using
immunocytochemistry.

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TABLE II

SUMMARY OF BDNF RESPONSIVE
AND NON-RESPONSIVE NEURONS*

A.   Responsive Neurons

    I.   Chick sensory neurons of neural crest origin in:

        (a) dorsal root ganglion

        (b) jugular ganglion

        (c) dorsomedial trigeminal ganglion

        (d) mesencephalic trigeminal nucleus**

    II. Chick sensory neurons of ectodermal placode origin in:

        (a)   nodose ganglion

        (b)   vestibular ganglion

        (c)   petrosal ganglion

        (d)   geniculate ganglion

        (e)   ventrolateral trigeminal ganglion

    III. Rat retinal ganglion cells

B.   Non-Responsive Neurons

    I. Chick and rat sympathetic neurons

    II. Chick parasympathetic ciliary neurons

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*   From Barde et al., 1987, Prog. Brain Res. 71:185-189

** See Davies et al., 1986, Nature 319:497-499

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3.   SUMMARY OF THE INVENTION

The present invention relates to nucleic acid sequences encoding brain derived neurotrophic factor (BDNF), to the substantially pure protein, peptide fragments or derivatives produced in quantity therefrom, and to

antibodies directed toward BDNF protein, peptide fragments, or derivatives. The present invention makes available, for the first time, sufficient quantities of BDNF to enable anti-BDNF antibody production and to support diagnostic and therapeutic applications of BDNF.

In various embodiments of the invention, the BDNF nucleic acids, proteins, peptides, derivatives, or antibodies of the invention may be utilized in methods for the diagnosis and treatment of a variety of neurologic diseases and disorders, and, in particular, in the diagnosis and treatment of sensory neuron disorders and retinal degeneration. Furthermore, in specific embodiments of the invention, BDNF nucleic acids or BDNF gene products may be used in the diagnosis and treatment of neuroblastoma tumor, Parkinson's disease, and Alzheimer's disease. BDNF gene products may also be used to facilitate incorporation of implants into nervous tissue or, alternatively, to promote nerve regeneration following damage by trauma, infarction, infection, or post-operatively, in additional specific embodiments of the invention.

The invention also relates to pharmaceutical compositions comprising effective amounts of BDNF gene products or, alternatively, antibodies directed toward BDNF gene products, which may be utilized in the diagnosis or treatment of a variety of neurological diseases and disorders.

In addition, by providing the full nucleotide sequence of BDNF, the present invention allows for the comparison of BDNF and NGF genes, thereby identifying homologous regions and defining a BDNF/NGF gene family. Accordingly, the present invention relates to a method for identifying additional members of the BDNF/NGF gene family. In a specific embodiment, the method of the invention is used to identify a novel, non-NGF, non-BDNF member of the BDNF/NGF gene family. The invention further provides for

additional members of the BDNF/NGF gene family identified
according to the disclosed method and to their gene
products.

3.1.   ABBREVIATIONS AND DEFINITIONS

5 BDNF   brain derived neurotrophic factor
   hBDNF   human BDNF
   CAT   choline acetyltransferase
   CNS   central nervous system
   DRG   dorsal root ganglia (ganglion)
10 EDTA   ethylene diamine tetraacetic acid
   NGF   nerve growth factor
   PBS   phosphate buffered saline
   PCR   polymerase chain reaction
   PNS   peripheral nervous system
15 SDS   sodium dodecyl sulfate
   Tris   tris(hydroxymethyl)aminomethane

4.   DESCRIPTION OF THE FIGURES

Figure 1.   Nucleotide sequence and deduced amino-acid
20   sequence of porcine prepro BDNF cDNA. The complete
            sequence of two overlapping cDNA clones is shown in
            this figure. The peptide sequences obtained from
            microsequencing (Table III) are underlined. The only
            consensus sequence for N-glycosylation is double
25   underlined. The start of the mature BDNF sequence is
            marked by a double carat.

Figure 2.   Sequence comparison between NGF and BDNF. Areas
            with more than two amino acids found at identical
30   positions are indicated. The sequences start with the
            first amino acids of the mature proteins and end with
            the last ones before the stop codons. Fifty-one amino
            acids are common to BDNF and the various NGFs (Schwarz

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et al., 1989, J. Neurochem. 52:1203-1209), including
the 6 cysteine residues.

Figure 3.   Autoradiograph of Southern blots of EcoRI cut
human, monkey, rat, mouse, dog, cow, rabbit, chicken,
5   and yeast genomic DNA, hybridized to ^{32}P-labeled NGF
and BDNF probes.

Figure 4.   Northern blot analysis. From each tissue, 20 μg
total RNA was applied per lane and hybridized with a
10   ^{32}P-labeled cRNA mouse BDNF probe. Note that a strong
signal can be seen at about 1.45 kB with brain tissue,
but none with the 7 other tissues analyzed.

Figure 5.   Sequence of human BDNF cDNA and deduced amino
15   acid sequence, and comparison of DNA sequences from
pig, rat, and chicken.

Figure 6.   BDNF expression plasmid PCMV1-pBDNF.

20 Figure 7.   (a) Results of ELISA determination of binding of
antisera to B5 peptide, using serial dilutions of
antisera.
        (b) Quantitation of binding of 1:500 dilution of
various antisera to 50 ng BDNF.
25

Figure 8.   Results of immunohistochemical staining for
tyrosine hydroxylase in BDNF-treated (hatched bars) and
control (solid bars) ventral mesencephalon cultures.

30
Figure 9.   Dopamine uptake by ventral mesencephalon
cultures. Cultures supplemented with BDNF are denoted
by hatched bars; control cultures are represented by
solid bars.

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Figure 10. (a) The effect of BDNF on the number of CAT positive cells in forebrain cholinergic neuron cultures.

(b) Forebrain cholinergic neuron cultures, at densities of 260,000 cells per well (black bar) or 150,000 cells per well (hatched bar), were treated with 150 ng/ml of NGF. The number of CAT immunopositive cells in NGF-treated versus untreated cells was compared.

Figure 11. Changes in the amount of CAT enzyme activity, in picomoles of substrate catalyzed per minute, as a function of BDNF concentration in forebrain cholinergic neuron cultures.

Figure 12. Astroglial cell cultures, at approximately 60% confluency, were treated with either (a) epidermal growth factor, or (b) BDNF for 42 hours, then incubated with [^{3}H] methylthymidine. Amount of ^{3}H incorporated was measured relative to EGF and BDNF concentration.

Figure 13. Southern blot of EcoRI restricted chicken, mouse, rat, hybridized to BDNF/NGF probe R1B/2C. The positions of the NGF and BDNF genomic EcoRI fragments are indicated as N and B, respectively.

Figure 14. Sequence comparison of NGF and BDNF with novel member of BDNF/NGF family identified by PCR from mouse DNA with Box 3/Box 4 primers: Novel gene [designated here as M3/4], also known as Neurotrophin-3 (NT-3), showing sequence of coding strand only, and deduced amino acid sequence aligned relative to mature mouse NGF and mature pig, rat, mouse, or human BDNF. Dashes indicate a position where a deletion of one codon in NGF is used to optimize alignment relative to BDNF and

M3/4.   Italics indicate matches in amino acid sequence
and/or conservative amino acid substitutions.

Figure 15.   Northern blots of RNA from a variety of human
            tumor-derived cell lines hybridized to BDNF human
5   probe.

Figure 16.   Effect of depolarization of BDNF-mRNA levels in
            hippocampal neurons.
                (a)   Time-course of BDNF-mRNA expression in
10   primary cultures of hippocampal neurons in the presence
            of 50mM KCl.   Total cellular RNA was extracted from 0.5
            x 10^6 cells, glyoxylated and analyzed by
            electrophoresis on a 1.3% agarose gel (Biziere, K. and
            Coyle, T., Neurosci., 1978, Lett. 8:303; McGeer et al.,
15   1978, Brain. Res. 139:381).   RNA transferred to Hybond
            N filters was hybridized with a ^{32}P-labelled cRNA probe
            (specific activity, 10^9 cpm/µg) specific for mouse BDNF
            and produced by run-off transcription in vitro.   The
            two upper bands (4 and 1.5kB) correspond to BDNF-mRNA;
20   the two bands for BDNF (4kb and 1.5 kb) are RNAse A-
            resistant and represent two different transcripts which
            seem, however, to be regulated in a similar manner) and
            the lower (700 bp) to 10 pg of a short BDNF-mRNA
            recovery standard which was added to samples before
25   extraction of RNA.
                (b)   Calcium dependency.   Neurons were incubated
            for 3 hours in normal culture medium (CM), in a modified
            medium without calcium (-Ca) or in culture medium with
            10µM nifedipine (NIF).   50mM KCl was added where
30   indicated (+K).

Figure 17.   Increases in NGF-mRNA levels in hippocampal
            neurons by potassium and kainic acid.   Neurons were
            incubated for 3 hours in control medium (C) or in the
35

presence of 50mM KCl (K+) or 25μM kainic acid (KA). RNA was extracted and the NGF-mRNA levels were determined by the quantitative PCR (11). The values are means ± SEM (n = 6).

5 Figure 18. Dose-response curve for the kainic acid effect. Hippocampal neurons were incubated for 3 hours in the presence of various concentrations of kainic acid. RNA was extracted and analyzed as described in Fig. 16. Values represent means ± SEM of three experiments.

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Figure 19. Time-course of increases in BDNF- and NGF-mRNA levels by kainic acid treatments. Total cellular RNA was extracted and analyzed as indicated in Fig. 16 from hippocampus (a) or cortex (b) at the indicated times

15 (in hours) after intraperitoneal injection of kainic acid (12mg/kg). One single dose of diazepam (10mg/kg) was injected 90 minutes after kainic acid (the two bands for BDNF (4kb and 1.5 kb) are RNAse A-resistant and represent two different transcripts which seem,

20 however, to be regulated in a similar manner). Values corresponding to 1.5 Kb BDNF-mRNA (o) and 1.3 Kb NGF-mRNA (o) are shown. Similar increases were observed for the 4Kb BDNF-mRNA. Values given represent means ± SEM of three to four experiments.

25

Figure 20. Effect of anticonvulsants on the kainic acid-induced expression of BDNF-mRNA. Rats were injected intraperitoneally with diazepam (10mg/kg) (DZ); MK-801 (1mg/kg (MK) or Ketamine (20mg/kg) (KET) 15 minutes

30 before injection of kainic acid (12mg/kg) (+KA) or saline (controls). Three hours later hippocampal tissue was used to prepare total RNA as indicated in Fig. 16. Values represent means ± SEM of three experiments.

35

Figure 21. Septal Cell Cultures.

A-F. Phase-Contrast Micrograph of the Time Course of
Cell Growth in Culture, The cells were plated at a
density of 1.3 x (10^5) cells/cm^2 and maintained in 5
HS/NS as described in the text. The time points
recorded were 1 (A, B), 2 (C, D) and 4 (E, F) days.
Cell type specific markers were used to identity
populations of cells, AChE histochemically stained
neurons (G, H) and NGF-receptor immunopositive neurons
(I). Scale bar = 25 μm.

Figure 22. Comparison of the Response of Dissociated Septal
Cells to BDNF or NGF Treatment on the Basis of AChE
Cell Numbers.

A. Comparison of the response to BDNF (25ng/ml), NGF
(50ng/ml) or the combination of both ligands at
different plating densities. (B) Comparison of the
response of cholinergic neurons to a range of BDNF or
NGF concentrations (0-50ng/ml). The cells for these
results were grown for a period of 11-12 days in
serum-containing medium. The data points represent the
mean $\pm$ S.E.M. of 6-9 determinations.

Figure 23. Bar Graph Depicts the Effect of Delaying the
Addition of BDNF or NGF to Dissociated Septal Cultures.
BDNF (50ng/ml) or NGF (50ng/ml) was added to
dissociated cell cultures following a delay of 5-6
hours (+12), 5 days (-5/+7) or 7 days (-7/+5). The
cultures were maintained for 12 days. Cells were
scored on the basis of AChE positive histochemical
staining. The results are expressed as the mean $\pm$
S.E.M. of 4 to 5 determinations.

Figure 24. Bar Graph Depicting the Ability of BDNF or NGF
to Regulate the Numbers of NGF-Receptor Immunopositive
Cells.

The immunoslating for the NGF-receptor was conducted
utilizing the monoclonal antibody 192-IgG at a dilution
of 1:1000. The dissociated septal cells were plated at
a density of 1.3 x (10^5) cells/cm^2, and treated
continuously for a period of 12 days. A comparison is
made between a saturating dose of NGF and a range of
doses of BDNF (0-100ng/ml). The results depicted are
the mean $\pm$ S.E.M. of an n of 4.

Figure 25. Dose Response Curve of Septal Cholinergic
Neurons to CAT Inducing Activities of BDNF (A) and NGF
(B).

Cultures were plated on polyornithine and laminin-
coated 6mm wells and maintained for 12 days in 5 HS/N3.
The cells were exposed to BDNF or NGF 5-6 hours after
plating. The factors were replaced every 3 days when
the medium was changed. The results shown represent
the mean $\pm$ S.E.M. of an n of 5-6 determinations.

Figure 26. Dose Response Effect of BDNF and NGF on AChE
Enzyme Activity.

Rat septal cholinergic neurons were grown for 12 days
in serum-containing medium with hormonal supplements.
The cells were treated with BDNF (open squares) and NGF
(solid squares) as described for Figure 25. The
results are the mean $\pm$ S.E.M. of an n of 6-9. The AChE
activity in the non-treated cultures was 16.4$\pm$2
nmol/Hr/Well.

Figure 27. Time Course of the Increase in CAT Enzyme
Activity Induced by BDNF as Compared to NGF.

The time requirement for the stimulation of CAT
activity was conducted on cells plated at a density of
$2.3 \times (10^5)$ cells/cm^2 and grown for varying lengths of
time in serum-containing medium with hormonal
supplements and exogenous factors. The cells were

5   initially exposed to BDNF (open squares) or NGF (solid
squares) 5-6 hours after plating. The results
represent the percent of the activity determined in the
treated cultures versus the non-treated cultures (n=6,
BDNF 12 days n=3).

10

Figure 28. Comparison of the Effects of Glial Cells on the
Ability of BDNF to Induce CAT Enzyme Activity.
Septal cells were plated at a density of $2.3 \times (10^5)$
cells/cm^2. Following a plating period of 5-6 hours,

15   the medium was changed to either serum-free or a
serum-containing medium both supplemented with 1% N3 as
detailed in the text. Cytosine arabinoside (1uM) was
added for 24 hours in order to further reduce the
numbers of astrocytes. The cultures were then

20   maintained for 12 days. The results represent the mean
$\pm$ S.E.M. of 4 determinations.

Figure 29. Bar Graph Depicts the Effect of BDNF or NGF
Treatment on the Level of High-Affinity Choline Uptake.

25   The choline uptake was conducted on cells maintained
for 11 days, following plating at a density of $1.3 \times$
(10^5) cells/cm^2. BDNF (50ng/ml) or NGF (50ng/ml) were
present during the entire culture period. The data
points represent the mean $\pm$ S.E.M. of 5 determinations

30   for BDNF and 10 for NGF.

Figure 30. Resolution by SDS page of ^{35}S-labeled reaction
products from the enzymatic cleavage of human brain-

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derived neurotrophic factor by trypsin and
endoproteinase Arg-C.

Figure 31.   Graphic representation of the DRG bioassay
5   comparing untreated CHO-hBDNF with endoproteinase-
              cleaved CHO-hBDNF. Neurite outgrowth is scored
              between 0 and 5 with a score of 5 representing maximal
              bioactivity.

Figure 32.   Phase-contrast (A) and bright-field
10   photomicrographs (B,C) of dissociated cultures of E14
              rat ventral mesencephalic cells stained after 9 days
              in culture with monoclonal antibodies to tyrosine
              hydroxylase (TH).
              A & B. Phase-contrast and bright-field photographs of
15   the same field show the low abundance of TH+ neurons
              in these cultures. Only a single TH+ neuron (arrows)
              is seen in a field containing many phase-bright
              neurons with long neurites. Few nonneuronal cells are
              evident, by morpholoogy or by staining for GFAP (not
20   shown).
              C. Bright field photograph showing two TH+ neurons
              (small arrows) in a field containing about ~ 98
              phase-bright cells of neuronal morphology. Scale bar
              = 100 1M.
25

Figure 33.   High magnification photomicrographs of 6 day
              cultures of E14 rat ventral mesencephalic cells
              showing varied morphologies of TH+ neurons, some with
              extensive neuritic growth and extremely elaborate
30   growth cones (A, B). Cultures were established and
              stained as described in legend to Fig. 32. Scale bar
              = 25 μM.

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Figure 34. BDNF promotes survival of TH+, dopaminergic
mesencephalic neurons in culture in dose-dependent
manner, evident at culture periods longer than 3 days.
A. Comparison of the number of TH+ neurons in
cultures of E14 rat ventral mesencaphalon maintained
5   with (solid bars) or without BDNF (open bars). In
treated cultures, BDNF (50 ng/ml) was added once on
culture day 2. No difference between control and
treated cultures was observed at day 3, but the number
of TH+ neurons in BDNF treated cultures was 1.8-fold
10   higher than controls at day 8. Values are the average
of duplicate samples.
B. Effect of BDNF on increasing survival of TH+
neurons is dose-dependent.
The number of TH+ neurons was determined in duplicate
15   cultures after 8 days in culture in the absence of
BDNF or medium containing increasing concentrations of
BDNF added at day 2.
C. NGF has no effect on the survival of TH+ neurons.

20   To assess the specificity of BDNF, cultures were also
grown in the presence or absence of NGF (50 ng/ml) for
8 days and analyzed for TH+ cells. NGF, added on day
2, did not increase the survival of TH+ neurons in
cultures examined at either day 6 or day 8. Results
25   are the average of duplicate dishes.

Figure 35. Repeated doses of BDNF further increase the
survival of TH+ neurons when compared to a single
addition of BDNF at day 2.
30   Cultures were prepared as described in the text.
Recombinant human BDNF was purified from the
supernatant of COS M5 cells. Cultures were treated
either with a single addition (SA; stipled bars) of
BDNF (50 ng/ml) on day 2, or with repeated doses of
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BDNF (50 ng/ml) on days 1, 2, 4, 6 and 8 (MA-multiple additions; solid bars), or were grown without BDNF (control; open bars). At the times indicated cultures were fixed and stained for TH immunoreactivity. Replenishing BDNF by multiple additions resulted in a

5   2.7 fold higher number of TH+ neurons at day 11 compared to controls, whereas the maximum increase seen with a single addition of BDNF was a little less than 2-fold after 11 days. Results, typical of several experiments, are the average of duplicate

10   samples.

Figure 36. Delayed addition of BDNF does not increase the number of TH+ neurons to the same extent as seen when BDNF is added at day 2.

15   Cultures were prepared as described in the text, with the exception of the time of addition of exogenous BDNF. All cultures were switched to serum-free medium at day 2, and BDNF (50 ng/ml, pig brain BDNF) was added as a single addition either at day 2, day 5 or

20   day 7 (CD2, CD5, CD7). At culture days six, eight and ten, the number of TH+ neurons was determined in duplicate cultures. In this experiment, a single addition of BDNF at day 2 resulted in a 3-fold higher number of TH+ neurons at day 10. When BDNF was added

25   after a delay, at day 5, an increase in TH+ neurons over controls was seen at day 10, but was less than 2-fold, and this difference in TH+ cell number between treated and control cultures did not increase further with longer time in culture. Control, open bars;

30   BDNF addition at day 2, stippled bars; BDNF added at day 5, solid bar; BDNF added at day 7, diagonal-hatched bars.

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Figure 37. Dose-dependent response of BDNF on high
            affinity GABA uptake in hippocampal cultures.
            Partially-purified nBDNF (rotophor-purified from COS
            M5 supernatants) was added to the cultures at various
            dilutions. BDNF at 1×10^{-2} dilution was found to
5   have maximal neurite-promoting activity in E8 chick
            dorsal root ganglion. High affinity ^{3}H-GABA uptake
            was measured following 8 days of BDNF treatment in
            vitro.

10 Figure 38. BDNF protects cultured nigral dopaminergic
            neurons from the neurotoxic effects of MPP+. Cultures
            were established from E14 rat embryos as described in
            Figure 1. After 24 hours in vitro the culture medium
            was changed to the serum-free formulation. After 3
15   days in culture the cultures were divided into four
            groups of six 35mm dishes. One group was kept as a
            control group, and the others received either (i)
            basic fibroblast growth factor, 10ng/ml (bovine brain
            bFGF; Boerhinger-Mannheim); (ii) mouse NGF, 50ng/ml;
20   or (iii) BDNF, 50ng/ml. Cultures were kept for a
            further 24 hours before 3 dishes in each group were
            exposed to 1μM MPP+ (Research Biochemicals, Inc.,
            Natick, MA) for 48 hours. At the end of the
            experiment, 6 days in total, all plates were processed
25   for TH immunoreactivity and the number of TH+ cells
            was determined in each group. The data presented
            represent the number TH+ neurons after MPP+ treatment
            calculated as a percentage of the number of TH+
            neurons in similar cultures not exposed to MPP+. All
30   values are the mean±s.e.m. of triplicate cultures.

            5.   DETAILED DESCRIPTION OF THE INVENTION
                 The present invention relates to nucleic acid
35   sequences encoding brain derived neurotrophic factor (BDNF),

as well as BDNF protein, peptide fragments and derivatives produced in quantity using these nucleic acid sequences. In addition, the invention relates to pharmacologic compositions and therapeutic uses of BDNF, having provided, for the first time, the means to generate sufficient quantities of substantially pure BDNF for clinical use. The invention also relates to antibodies directed toward BDNF or fragments thereof, having provided a method for generating sufficient immunogen. Further, by permitting a comparison of the nucleic acid sequences of BDNF and NGF, the present invention provides for the identification of homologous regions of nucleic acid sequence between BDNF and NGF, thereby defining a BDNF/NGF gene family; the invention provides a method for identifying and isolating additional members of this gene family.

For purposes of clarity of description, and not by way of limitation, the invention will be described in the following parts:

(i)   purification of BDNF
(ii)   BDNF bioassays
(iii)   microsequencing of BDNF protein
(iv)   cloning of BDNF encoding DNA
(v)   expression of BDNF
(vi)   BDNF genes and proteins
(vii)   generation of anti-BDNF antibodies
(viii) identification of additional members of the BDNF/NGF gene family
(ix)   utility of the invention
(x)   pharmaceutical compositions

5.1. PURIFICATION OF BRAIN DERIVED NEUROTROPHIC FACTOR

In order to identify BDNF encoding nucleic acid, microgram amounts of BDNF protein may be obtained from tissue to permit the determination of amino acid sequences

which may then be used to design oligonucleotide probes. The extreme rarity of BDNF protein practically limits the amount of amino acid sequence which may be determined. BDNF may be prepared from pig brain by methods described in Barde et al., (1982, EMBO J. 1:549-553), or Hofer and Barde (1988,

5   Nature 331:261-262).

        Preferably, BDNF may be prepared, according to the following detailed protocol which is presented by way of example, and not by way of limitation; various modifications are envisioned for use by one skilled in the art. Because

10 of the rarity of BDNF, preferably about six kilograms of brain tissue may be used in a purification procedure. Brain tissue may be homogenized in sodium phosphate buffer at a concentration of about 0.2 M sodium phosphate and a pH of approximately 6, containing 1 mM EDTA and 1 mM freshly added

15 phenylmethanesulphonyl fluoride, such that the ratio of brain tissue to fluid is approximately 1 kg of brain to 2 liters of buffer. Hydrochloric acid may then be used to adjust the pH of the mixture to approximately 4, and the mixture may then be stirred for about 2 hours at 4°C. The

20 mixture can then be centrifuged for 25 minutes at 20,000g. After centrifugation, the supernatant is collected, adjusted to a pH of about 6.0 using sodium hydroxide, and then stirred with about 1 liter of preswollen carboxymethylcellulose (per 6 kg of brain tissue) which has

25 been equilibrated with 0.1 M sodium phosphate at a pH of 6. After several washes with a total of about 20 liters of 0.1 M sodium phosphate, pH 6, the slurry may be poured into a column and washed with the same buffer containing 0.13 M NaCl, preferably overnight. Active fractions, identified by

30 a BDNF sensitive bioassay (see Section 5.2, infra) may then be eluted with phosphate buffer containing 0.5 M NaCl and subsequently dialyzed against several changes of about 5 liters of 5 mM potassium phosphate at a pH of 6.8. The dialyzed fractions may then be applied to a hydroxyapatite

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column having a bed volume of about 20 ml for each kilogram
of brain tissue processed; the hydroxyapatite column should
be pre-equilibrated with 5 mM potassium phosphate at a pH of
6.8 prior to application of sample. The column may then be
eluted with a linear gradient composed of 500 ml of 5 mM
5 potassium phosphate and 500 ml of 700 mM potassium
phosphate, both at a pH of about 6.8. The BDNF activity
should optimally be eluted at approximately 500 mM potassium
phosphate. Pooled active fractions may then be adjusted to
a final molarity of about 700 mM potassium phosphate and
10 applied to a phenyl-sepharose column (having a volume of
about 5 ml for every 6 kg of brain tissue processed)
equilibrated with a solution of 700 mM potassium phosphate
having a pH of 6.8. After washing with approximately 40 ml
of the same buffer, BDNF activity may be eluted with 0.1 M
15 potassium phosphate, pH 6.8, and subsequently the BDNF
activity may be dialyzed against distilled water, and then
lyophilized. The lyophilized material may then be taken up
in an SDS-gel electrophoresis sample buffer containing 0.1%
SDS but preferably containing no mercaptoethanol, and
20 applied to an SDS gel comprised of a linear gradient of 10-
25% acrylamide. After completion of electrophoretic
separation, the gel may be stained for about 10 minutes with
Coomassie blue, and destained for about 20 minutes. A band
migrating at the level of a cytochrome c marker may be cut
25 out and electrophoretically eluted from the gel. SDS may be
largely removed as described by Weber and Kuter (1971, J.
Biol. Chem. 246:4504-4509). It should be noted that removal
of SDS is generally not complete. A purification procedure
for microsequencing of BDNF was modified according to Hofer
30 and Barde (1988, Nature 331:261-262), and does not make use
of a gel electrophoresis as the last step of purification.

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5.2.   BRAIN DERIVED NEUROTROPHIC FACTOR BIOASSAYS

Any system which qualitatively or quantitatively indicates BDNF activity may be used according to the present invention.   Such bioassays may be useful in identifying and/or measuring natural or recombinant BDNF activity.

5   Any BDNF bioassay known in the art may be used. For example, chick embryo dorsal root ganglion (DRG) neurons may be utilized in a BDNF assay, as described in Barde et al. (1980, Proc. Natl. Acad. Sci. U.S.A. 77:1199-1203).

By way of example, dorsal root ganglia from 6 to
10   14 day chick embryos, and preferably 10 to 12 day chick embryos, may be collected, using dissection techniques known in the art, and immediately placed in a small volume of F14 medium (made from GIBCO F-12 powder, supplemented as in Vogel et al., 1972, Proc. Natl. Acad. Sci. U.S.A. 69:3180-
15   3184), which should be replaced at the end of the dissection by Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS) containing about 0.1 percent trypsin.   After about 20 minutes of incubation at 37°C, the ganglia may be centrifuged and washed twice with F14 medium containing
20   about 10 percent (vol/vol) heat-inactivated horse serum. The ganglia may then be dissociated by gentle trituration (about 10-15 aspirations) using a small (about 1 mm) diameter siliconized pasteur pipette.   Remaining lumps of tissue may then, preferably, be removed by passing the cell
25   suspension through a nylon mesh, having about 40 μm sized pores.   The cell suspension may then be preplated for about 210 minutes on a plastic tissue culture dish; during this period, most of the nonneuronal cells should adhere to the plastic surface, leaving a neuron-enriched cell population
30   in suspension.   Cells may then be diluted to a concentration of about 5 x 10³ cells per milliliter of plating medium (preferably F14 medium supplemented with 10 percent vol/vol heat inactivated horse serum together with antibiotics) and placed in polyornithine or, preferably,
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polyornithine/laminin coated tissue culture dishes (see
infra).

Alternatively, and not by way of limitation, BDNF
bioassays which are relatively insensitive to NGF may, in
some circumstances, be preferable to systems, such as the
DRG system described _supra_, which may, under certain
conditions, respond to both BDNF and NGF. Such relatively
BDNF-specific systems would include retinal ganglion
cultures as well as cultures of neurons derived from neural
placodes.

Perinatal retinal cells may be cultured according
to the methods described in Johnson et al. (1986, J.
Neurosci. 6:3031-3038). For example, retinas may be removed
from perinatal animals (in the rat the term "perinatal" here
refers to embryonic day 17 embryos through postnatal pups
within 48 hours after birth), washed in calcium and
magnesium free phosphate buffered saline (PBS), then
incubated in PBS containing about 0.05% to 0.1% trypsin for
approximately 15 minutes at about 37°C. After proteolytic
digestion, retinas may be washed in F14 culture medium
containing about 10 per cent (vol/vol) heat-inactivated
horse serum (F14-HS). The retinas may then be dissociated
by gentle pipetting in a small volume (about 1-10 ml) of
fresh F14-HS. Undissociated tissue may be allowed to
settle, and the remaining cells and medium pipetted off for
culture.

Alternatively, BDNF bioassays which comprise
neural placode derived cells may be used according to the
invention. Embryonic cranial nerve explants of, for
example, the ventrolateral portion of the trigeminal
ganglion, or the vestibular, geniculate, petrosal, or nodose
ganglion may be cultured in collagen-gel overlaid with
culture medium, as described in Davies et al. (1986, J.
Neurosci. 6:1897-1904) or dissociated according to Barde et
al. (1980, Proc. Natl. Acad. Sci. 77:1199-1203).

Because it has been observed that responsiveness
to BDNF may be increased ten-fold by changing the growth
substrate from polyornithine to laminin-polyornithine,
(Barde et al., 1987, Prog. Brain Res. 71:185-189), BDNF
assays are preferably performed on laminin-containing
5 substrates. Culture surfaces may be prepared, for example,
by (i) coating the culture surface for about 8-10 hours with
a sterile solution of polyornithine; (ii) washing several
times with sterile water; and (iii) coating the culture
surface for about two hours with laminin at a concentration
10 of approximately 25 μg/ml in PBS (Johnson et al., 1986, J.
Neurosci. 6:3031-3038).

In any bioassay system used according to the
invention, a BDNF dose-response curve may be established
using methods known in the art. Using a dissociated chick
15 sensory ganglion assay, half-maximal survival was observed
with a concentration of about 5 ng/ml purified BDNF, and
maximal survival was observed with a concentration of
between about ten and 20 ng/ml purified BDNF (Barde et al.,
1987, Prog. Brain Res. 71:185-189).
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5.3. MICROSEQUENCING OF BRAIN DERIVED NEUROTROPHIC FACTOR PROTEIN

BDNF protein prepared from brain may be sequenced;
however, it must be emphasized that the extreme rarity of
25 the protein makes it unlikely that a substantial portion of
BDNF protein sequence may be reliably obtained. The
protein may be sequenced directly or initially cleaved by
any protease or other compound known in the art, including,
but not limited to, Staphylococcus aureus V8, trypsin, and
30 cyanogen bromide. Peptides may be sequenced by automated
Edman degradation on a gas phase microsequencer according to
the method of Hewick et al. (1981, J. Biol. Chem. 256:7990-
7997) and Hunkapillar et al. (1983, Methods Enzymol.
91:227-236). Detection of phenylthiohydantoin amino acids
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may then be performed according to Lottspeich (1985, Chromatography 326:321-327). Overlapping fragments of amino acid sequence may be determined and used to deduce longer stretches of contiguous sequence.

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5.4. CLONING OF BRAIN DERIVED NEUROTROPHIC FACTOR-ENCODING DNA

The rarity of BDNF effectively precludes the use of standard strategies for cloning the BDNF gene. For example, if available protein sequence were used to generate

10 a complementary labeled oligonucleotide probe, and this probe were used to screen cDNA libraries generated from tissue presumed to synthesize BDNF, the number of positive clones would be likely to be vanishingly small. The instant invention provides for the cloning of the BDNF gene by a

15 combination of procedures, comprising the purification of suitable amounts of BDNF protein, microsequencing of the BDNF protein, derivation of an oligonucleotide probe, construction of a cDNA library, amplification based on the derived BDNF amino acid sequence, and finally, selection for

20 the BDNF gene. In this method of the invention, the preferred procedure for amplification utilizes the amplification of tissue nucleic acid sequences by polymerase chain reaction (PCR) (Saiki et al., 1985, Science 230:1350-1354), in order to expand the number of BDNF sequences

25 available for cloning. A detailed description of the preferred method follows:

Firstly, the amino acid sequence derived from purified BDNF protein may be used to deduce oligonucleotide primers for use in polymerase chain reaction. Because of

30 the degeneracy of the genetic code, in which several nucleic acid triplets may specify the same amino acid, several oligonucleotides should be synthesized for a given amino acid sequence, in order to provide for multiple potential

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nucleotide sequence combinations; the resulting
oligonucleotides are referred to as degenerate primers.

The polymerase chain reaction (PCR) requires sense
strand as well as anti-sense strand primers. Accordingly, a
degenerate oligonucleotide primer corresponding to BDNF
5   amino acid sequence may be used as primer for one DNA
strand, and a second primer, homologous to a commonly
occurring DNA sequence, such as a stretch of thymidine
residues resulting from reverse transcription of the
polyadenosine tail of mRNAs, may be used as primer for the
10 second DNA strand. These primers may then be used in
polymerase chain reaction with nucleic acid template
presumed to contain BDNF encoding sequences, such as genomic
DNA or, preferably, cDNA prepared from mRNA collected from
tissue presumed to synthesize BDNF. The DNA reaction
15 products may then be analyzed by electrophoresis to
determine whether the DNA reaction product has a molecular
size similar to the expected size of the BDNF gene and,
preferably, by nucleotide sequence analysis.

However, because the use of two degenerate primers
20 in PCR increases the likelihood of amplifying nucleic acid
sequences which do not encode BDNF, a preferred method
provides for the use of only one degenerate primer, the
other primer corresponding to exact BDNF sequence. In order
to identify exact BDNF sequence, the amino acid sequence
25 determined using purified BDNF protein may be used to design
both degenerate sense and anti-sense oligonucleotide
primers. The DNA reaction product resulting from the use of
these primers in PCR reaction, using BDNF encoding nucleic
acid as a template, should be a nucleic acid fragment
30 encoding the stretch of amino acids used to design the
primers, and may be of a predictable size (e.g. at minimum,
the number of amino acid residues, multiplied by a factor of
three, base pairs in length). Sequence analysis of the DNA
reaction product may be compared to the ascertained amino
35

acid sequence to corroborate that the amplified nucleic acid
sequence may, in fact, encode the BDNF peptide fragment.
Although any method of nucleic acid sequencing known in the
art may be utilized, it is preferable to use the
dideoxynucleotide chain termination method (Sanger et al.,
1979, Proc. Natl. Acad. Sci. U.S.A. 72:3918-3921).
Sequencing may be accomplished using gel purified or,
preferably, cloned DNA reaction product. The sequence of
the DNA reaction product may then be used toward designing
an oligonucleotide primer corresponding to exact BDNF-
encoding sequence. This primer may then be used together
with a second primer, which may be degenerate, to extend the
amount of BDNF-sequence beyond that represented by the
fragment of exact sequence initially determined. For
example, and not by way of limitation, the sense strand
primer may correspond to exact BDNF nucleotide sequence,
whereas the anti-sense primer may be a degenerate primer
homologous to a region of sequence likely to be found
downstream of the sequenced fragment, e.g. the polyadenosine
tail of mRNA, as reverse transcribed in the cDNA. It may
then be necessary to use a similar method to retrieve
sequence upstream of the sequenced fragment; for example,
the anti-sense strand primer may correspond to exact BDNF
nucleotide sequence and the sense strand primer may be a
degenerate primer homologous to a region of BDNF sequence
upstream of the sequenced fragment, e.g. a 5' polyadenosine
tail added at the 5' end of cDNA using terminal
deoxynucleotide transferase. Accordingly, the sequence of
the entire BDNF gene or mRNA may be assembled.

        DNA reaction products may be cloned using any
method known in the art. A large number of vector-host
systems known in the art may be used. Possible vectors
include, but are not limited to, cosmids, plasmids or
modified viruses, but the vector system must be compatible
with the host cell used. Such vectors include, but are not

limited to, bacteriophages such as lambda derivatives, or
plasmids such as pBR322, pUC, or Bluescript® (Stratagene)
plasmid derivatives. Recombinant molecules can be
introduced into host cells via transformation, transfection,
infection, electroporation, etc.

5   The BDNF gene is inserted into a cloning vector
which can be used to transform, transfect, or infect
appropriate host cells so that many copies of the gene
sequences are generated. This can be accomplished by
ligating the DNA fragment into a cloning vector which has

10   complementary cohesive termini. However, if the
complementary restriction sites used to fragment the DNA are
not present in the cloning vector, the ends of the DNA
molecules may be enzymatically modified. It may prove
advantageous to incorporate restriction endonuclease

15   cleavage sites into the oligonucleotide primers used in
polymerase chain reaction to facilitate insertion into
vectors. Alternatively, any site desired may be produced by
ligating nucleotide sequences (linkers) onto the DNA
termini; these ligated linkers may comprise specific

20   chemically synthesized oligonucleotides encoding restriction
endonuclease recognition sequences. In an alternative
method, the cleaved vector and BDNF gene may be modified by
homopolymeric tailing.

          In specific embodiments, transformation of host
25   cells with recombinant DNA molecules that incorporate an
isolated BDNF gene, cDNA, or synthesized DNA sequence
enables generation of multiple copies of the gene. Thus,
the gene may be obtained in large quantities by growing
transformants, isolating the recombinant DNA molecules from

30   the transformants and, when necessary, retrieving the
inserted gene from the isolated recombinant DNA.

          According to a preferred embodiment of the
invention, once a cDNA-derived clone encoding BDNF has been
generated, a genomic clone encoding BDNF may be isolated

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using standard techniques known in the art. For example, a labeled nucleic acid probe may be derived from the BDNF clone, and used to screen genomic DNA libraries by nucleic acid hybridization, using, for example, the method set forth in Benton and Davis (1977, Science 196:180) for
5 bacteriophage libraries and Grunstein and Hogness (1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961-3965) for plasmid libraries. Retrieved clones may then be analyzed by restriction-fragment mapping and sequencing techniques according to methods well known in the art.
10   Furthermore, additional cDNA clones may be identified from a cDNA library using the sequences obtained according to the invention.

           5.5.   EXPRESSION OF THE BRAIN DERIVED
15   NEUROTROPHIC FACTOR GENE
           The nucleotide sequence coding for a BDNF protein, or a portion thereof, can be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of
20 the inserted protein-coding sequence. The necessary transcriptional and translation signals can also be supplied by the native BDNF gene and/or its flanking regions. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not
25 limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA. The
30 expression elements of these vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

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        Any of the methods previously described for the
insertion of DNA fragments into a vector may be used to
construct expression vectors containing a chimeric gene
consisting of appropriate transcriptional/translational
control signals and the protein coding sequences. These
5 methods may include in vitro recombinant DNA and synthetic
techniques and in vivo recombinations (genetic
recombination). Expression of nucleic acid sequence
encoding BDNF protein or peptide fragment may be regulated
by a second nucleic acid sequence so that BDNF protein or
10 peptide is expressed in a host transformed with the
recombinant DNA moleucle. For example, expression of BDNF
may be controlled by any promoter/enhancer element known in
the art. Promoters which may be used to control BDNF
expression include, but are not limited to, the SV40 early
15 promoter region (Bernoist and Chambon, 1981, Nature
290:304-310), the promoter contained in the 3' long terminal
repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell
22:787-797), the herpes thymidine kinase promoter (Wagner et
al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the
20 regulatory sequences of the metallothionine gene (Brinster
et al., 1982, Nature 296:39-42); prokaryotic expression
vectors such as the β-lactamase promoter (Villa-Kamaroff, et
al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or
the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad.
25 Sci. U.S.A. 80:21-25), see also "Useful proteins from
recombinant bacteria" in Scientific American, 1980, 242:74-
94; plant expression vectors comprising the nopaline
synthetase promoter region (Herrera-Estrella et al., Nature
303:209-213) or the cauliflower mosaic virus 35S RNA
30 promoter (Gardner, et al., 1981, Nucl. Acids Res. 9:2871),
and the promoter for the photosynthetic enzyme ribulose
biphosphate carboxylase (Herrera-Estrella et al., 1984,
Nature 310:115-120); promoter elements from yeast or other
fungi such as the Gal 4 promoter, the ADC (alcohol
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dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phophatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the bran (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Expression vectors containing BDNF gene inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the

first approach, the presence of a foreign gene inserted in
an expression vector can be detected by DNA-DNA
hybridization using probes comprising sequences that are
homologous to an inserted BDNF gene. In the second
approach, the recombinant vector/host system can be
5 identified and selected based upon the presence or absence
of certain "marker" gene functions (e.g., thymidine kinase
activity, resistance to antibiotics, transformation
phenotype, occlusion body formation in baculovirus, etc.)
caused by the insertion of foreign genes in the vector. For
10 example, if the BDNF gene is inserted within the marker gene
sequence of the vector, recombinants containing the BDNF
insert can be identified by the absence of the marker gene
function. In the third approach, recombinant expression
vectors can be identified by assaying the foreign gene
15 product expressed by the recombinant. Such assays can be
based, for example, on the physical or functional properties
of the BDNF gene product in bioassay systems as described
supra, in Section 5.2.

        Once a particular recombinant DNA molecule is
20 identified and isolated, several methods known in the art
may be used to propagate it. Once a suitable host system
and growth conditions are established, recombinant
expression vectors can be propagated and prepared in
quantity. As previously explained, the expression vectors
25 which can be used include, but are not limited to, the
following vectors or their derivatives: human or animal
viruses such as vaccinia virus or adenovirus; insect viruses
such as baculovirus; yeast vectors; bacteriophage vectors
(e.g., lambda), and plasmid and cosmid DNA vectors, to name
30 but a few.

        In addition, a host cell strain may be chosen
which modulates the expression of the inserted sequences, or
modifies and processes the gene product in the specific
fashion desired. Expression from certain promoters can be
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elevated in the presence of certain inducers; thus, expression of the genetically engineered BDNF protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g.,
5   glycosylation, cleavage) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein
10 product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of the heterologous BDNF protein. Furthermore, different vector/host expression systems may effect processing reactions such as proteolytic
15 cleavages to different extents.

In a specific embodiment of the invention, DNA encoding preproBDNF may be cloned into pCMV plasmid, amplified, and then used to transfect COS cells by the calcium phosphate method (Chen and Okayama, 1987, Mol. Cell.
20 Biol. 7:2745-2752); BDNF activity may then be collected from cell culture medium (see Example Section 10, infra).

5.5.1. IDENTIFICATION AND PURIFICATION OF THE EXPRESSED GENE PRODUCT

25   Once a recombinant which expresses the BDNF gene is identified, the gene product should be analyzed. This can be achieved by assays based on the physical or functional properties of the product.

Once the BDNF protein is identified, it may be
30 isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. The functional properties may be
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evaluated using any known BDNF assay, including, but not
limited to, chick embryo dorsal root ganglia, perinatal rat
retinal cells, or neurol placode-derived neurons.

        Importantly, methods used to prepare BDNF from
brain tissue, because they involve, as a final step,
5   preparative gel electrophoresis, would produce BDNF which
was not fully active due to the presence of residual SDS
(Barde and Thoenen, 1985, in "Hormones and Cell Regulation,"
Vol. 9, Dumont et al., eds., Elsevier Science Publishers,
pp. 385-390). In contrast, the present invention permits
10 the isolation of BDNF which is produced from recombinant
nucleic acid molecules and which is free of SDS therefore
possesses full activity. For example, and not by way of
limitation, the anti-BDNF antibodies of the invention (such
as antibody directed toward the B5-33 amino acid fragment of
15 porcine BDNF, described in Section 11, infra) may be used to
collect recombinant BDNF by immunoprecipitation or affinity
chromatography, thereby producing detergent-free, fully
active BDNF.

        In a further embodiment of the invention, prepro
20 BDNF may be converted to active mature BDNF enzymatically,
using, for example, endoproteinase Arg-C (see Example
Section 17, infra).

                5.6.   BRAIN DERIVED NEUROTROPHIC FACTOR
25   GENES AND PROTEINS
        Using the methods detailed supra and in Example
Sections 6 and 9, infra, the following nucleic acid
sequences were determined, and their corresponding amino
acid sequences deduced. The porcine BDNF cDNA sequence was
30 determined, and is depicted in Figure 1. The human genomic
cDNA BDNF sequence was determined, and is depicted in Figure
5 which also presents DNA sequences from pig, rat, and
chicken. Each of these sequences, or their functional
equivalents, can be used in accordance with the invention.
35

Additionally, the invention relates to BDNF genes and proteins isolated from porcine, bovine, feline, avian, equine, or canine, as well as primate sources and any other species in which BDNF activity exists. The invention is further directed to subsequences of BDNF nucleic acids

5   comprising at least ten nucleotides, such subsequences comprising hybridizable portions of the BDNF sequence which have use, e.g., in nucleic acid hybridization assays, Southern and Northern blot analyses, etc. The invention also provides for BDNF protein, and fragments and

10 derivatives thereof, according to the amino acid sequences set forth in Figures 1 and 5 or their functional equivalents. The invention also provides fragments or derivatives of BDNF proteins which comprise antigenic determinant(s) or which are functionally active. As used

15 herein, functionally active shall mean having positive activity in assays for known BDNF function, e.g. chick embryo DRG assays.

For example, the nucleic acid sequences depicted in Figures 1 and 5 can be altered by substitutions,

20 additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as depicted in Figures 1 and 5 may be used in the practice of the present

25 invention. These include but are not limited to nucleotide sequences comprising all or portions of the BDNF genes depicted in Figures 1 and 5 which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus

30 producing a silent change. Likewise, the BDNF proteins, or fragments or derivatives thereof, of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence

35 substantially as depicted in Figures 1 and 5 including

altered sequences in which functionally equivalent amino
acid residues are substituted for residues within the
sequence resulting in a silent change. For example, one or
more amino acid residues within the sequence can be
substituted by another amino acid of a similar polarity
5   which acts as a functional equivalent, resulting in a silent
alteration. Substitutes for an amino acid within the
sequence may be selected from other members of the class to
which the amino acid belongs. For example, the nonpolar
(hydrophobic) amino acids include alanine, leucine,
10 isoleucine, valine, proline, phenylalanine, tryptophan and
methionine. The polar neutral amino acids include glycine,
serine, threonine, cysteine, tyrosine, asparagine, and
glutamine. The positively charged (basic) amino acids
include arginine, lysine and histidine. The negatively
15 charged (acidic) amino acids include aspartic acid and
glutamic acid. Also included within the scope of the
invention are BDNF proteins or fragments or derivatives
thereof which are differentially modified during or after
translation, e.g., by glycosylation, proteolytic cleavage,
20 linkage to an antibody molecule or other cellular ligand,
etc.

        Additionally, a given BDNF can be mutated in vitro
or in vivo, to create and/or destroy translation,
initiation, and/or termination sequences, or to create
25 variations in coding regions and/or form new restriction
endonuclease sites or destroy preexisting ones, to
facilitate further in vitro modification. Any technique for
mutagenesis known in the art can be used, including but not
limited to, in vitro site-directed mutagenesis (Hutchinson,
30 et al., 1978, J. Biol. Chem. 253:6551), use of TAB® linkers
(Pharmacia), etc.

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5.7.   GENERATION OF ANTI-BRAIN DERIVED NEUROTROPHIC FACTOR ANTIBODIES

According to the invention, BDNF protein, or fragments or derivatives thereof, may be used as immunogen to generate anti-BDNF antibodies. Previous attempts to

5 produce an anti-BDNF immune response have been unsuccessful, possibly because of the small quantities of purified BDNF available. By providing for the production of relatively abundant amounts of BDNF protein using recombinant techniques for protein synthesis (based upon

10 the BDNF nucleic acid sequences of the invention), the problem of insufficient quantities of BDNF has been obviated.

To further improve the likelihood of producing an anti-BDNF immune response, the amino acid sequence of BDNF

15 may be analyzed in order to identify portions of the molecule which may be associated with increased immunogenicity. For example, the amino acid sequence may be subjected to computer analysis to identify surface epitopes, according to the method of Hopp and Woods (1981,

20 Proc. Natl. Acad. Sci. U.S.A. 78:3824-3828) which has been successfully used to identify antigenic peptides of Hepatitis B surface antigen. Alternatively, the deduced amino acid sequences of BDNF from different species could be compared, and relatively non-homologous regions

25 identified; these non-homologous regions would be more likely to be immunogenic across various species.

For preparation of monoclonal antibodies directed toward BDNF, any technique which provides for the production of antibody molecules by continuous cell lines

30 in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma

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technique to produce human monoclonal antibodies (Cole et al., 1985, in "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc. pp. 77-96) and the like are within the scope of the present invention.

5   The monoclonal antibodies for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:7308-7312; Kozbor et al., 1983, Immunology Today

10 4:72-79; Olsson et al., 1982, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851, Takeda et al., 1985, Nature 314:452).

15   Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of BDNF. For the production of antibody, various host animals can be immunized by injection with BDNF protein, or fragment or derivative thereof, including but not limited to rabbits,

20 mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols,

25 polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and, Corynebacterium parvum.

       A molecular clone of an antibody to a BDNF epitope

30 can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to construct nucleic

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acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high

5   performance liquid chromatography), or a combination thereof, etc.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the

10 $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the 2 Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a

15 reducing agent.

Example Section 11 describes the preparation of polyclonal antisera directed toward the B5-33 peptide fragment of BDNF protein.

20

5.8.   IDENTIFICATION OF ADDITIONAL MEMBERS OF THE BDNF/NGF GENE FAMILY

Sequence analysis of the gene encoding BDNF and deduction of its amino acid sequence revealed that this protein has many structural similarities to NGF (Figure 2).

25 The primary sequence of mature BDNF, as well as the general structure and probable mode of processing from a precursor protein, suggest strongly that the NGF and BDNF genes may have evolved from a common ancestral gene. Within the region of the mature polypeptides, if only three gaps are

30 introduced in the NGF sequences to optimize matching, a total of 51 amino acid identities are common to the previously known NGFs from many species and to porcine and human BDNF. These identities include all six cysteine residues, suggesting that the NGFs and BDNF share very

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similar secondary structure.   Furthermore, four segments
of six or more amino acids can be seen in which NGFs from
all of the species listed above and from porcine BDNF are
either identical, or differ by no more than about one
conservative amino acid substitution.   Thus, it is
5 reasonable to conclude that NGF and BDNF appear to be
closely related members of a gene family.

A rational search for additional members of the
BDNF/NGF gene family may be carried out using an approach
that takes advantage of the unanticipated existence of the
10 conserved segments of strong homology between NGF and BDNF.
For example, additional members of the BDNF gene family may
be identified by selecting, from among a diversity of
nucleic acid sequences, those sequences that are homologous
to BDNF and NGF, and further identifying, from among the
15 selected sequences, those that also contain nucleic acid
sequences which are non-homologous to NGF and BDNF.   The
term "non-homologous" may be construed to mean a region
which contains at least about 6 contiguous nucleotides in
which at least about two nucleotides differ from NGF and
20 BDNF sequence.

A preferred embodiment of the invention provides
the following method.   Corresponding to each of the four
conserved segments ("boxes") described above and set forth
in Table III, infra, sets of degenerate oligonucleotide
25 probes of at least 18 nucleotides may be synthesized,
representing all of the possible coding sequences for the
amino acids found in either NGF or BDNF over six contiguous
codons.   Numbering with respect to the amino terminus of
the mature polypeptides (so that His134 of preproBDNF is
30 treated as His1 in the mature protein), the four boxes may
be characterized as follows (numbered relative to human
mature proteins; DNA sequence as depicted in Figure 1).

35

TABLE III

					DNA Sequence
Box 1:	NGF	Gly10	-	Ser19	
	BDNF	Gly8	-	Ser17	587-616
Box 2:	NGF	Lys50	-	Cys58	
	BDNF	Lys50	-	Cys58	713-739
Box 3:	NGF	Gly67	-	Asp72	
	BDNF	Gly67	-	Asp72	764-781
Box 4:	NGF	Trp99	-	Cys110	
	BDNF	Trp100	-	Cys111	863-898

Synthetic oligonucleotides derived from sequence pairs from the boxes set forth in Table III may be utilized as primers to amplify by PCR sequences from a source (RNA or DNA) of potential interest. This might include mRNA or cDNA or genomic DNA from any eukaryotic species that could express a polypeptide closely related to BDNF or NGF. By carrying out as few as six PCR reactions (namely: a primer from Box 1 with a primer from Box 2; Box 1 with Box 3; Box 1 with Box 4; Box 2 with Box 3; Box 2 with Box 4; Box 3 with Box 4), it may be possible to detect a gene or gene product sharing any two of the four above segments of conserved sequence between NGF and BDNF. If one chooses to synthesize several different degenerate primers for each box, it may still be possible to carry out a complete search with a reasonably small number of PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser degrees of nucleotide sequence similarity between the unknown gene and NGF or BDNF. If a segment of a previously unknown member of the BDNF/NGF gene family is amplified successfully, that segment may be molecularly cloned and sequenced, and utilized as a probe to isolate a

complete cDNA or genomic clone. This, in turn, will permit
the determination of the unknown gene's complete nucleotide
sequence, the analysis of its expression, and the
production of its protein product for functional analysis.

5   The approach described above has been used to
identify a novel gene related to both BDNF and NGF, and
described in Example Section 13, infra.

In addition, the present invention provides for
the use of the BDNF/NGF sequence homologies in the design
of novel recombinant molecules which are members of the
10   BDNF/NGF gene family but which may not occur in nature.
For example, and not by way of limitation, a recombinant
molecule can be constructed according to the invention,
comprising portions of both NGF and BDNF genes. Such a
molecule could exhibit properties associated with both BDNF
15   and NGF and portray a novel profile of biological
activities, including agonists as well as antagonists.
Primary sequence of BDNF and NGF may also be used to
predict tertiary structure of the molecules using computer
simulation (Hopp and Woods, 1981, Proc. Natl. Acad. Sci.
20   U.S.A. 78:3824-3828); BDNF/ NGF chimeric recombinant genes
could be designed in light of correlations between tertiary
structure and biological function. Likewise, chimeric
genes comprising portions of any one or more members of
BDNF/NGF gene family, including the novel member described
25   in Section 13 may be constructed.

             5.9. UTILITY OF THE INVENTION
             The present invention relates to the nucleic
acid sequence of BDNF and to the substantially pure
30   protein, peptide fragments, or derivatives produced
therefrom. BDNF may be produced, for the first time, in
quantities sufficient for diagnostic and therapeutic
applications. Likewise, anti-BDNF antibodies, also
available for the first time as a consequence of the
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invention, and BDNF nucleic acid probes, may be utilized in
diagnostic and therapeutic applications. For most
purposes, it is preferable to use BDNF genes or gene
products from the same species for diagnostic or
therapeutic purposes, although cross-species utility of
5 BDNF may be useful in specific embodiments of the
invention.

5.9.1.   DIAGNOSTIC APPLICATIONS

         The present invention, which relates to nucleic
10 acids encoding BDNF and to proteins, peptide fragments, or
derivatives produced therefrom, as well as antibodies
directed against BDNF protein, peptides, or derivatives,
may be utilized to diagnose diseases and disorders of the
nervous system which may be associated with alterations in
15 the pattern of BDNF expression.

         In various embodiments of the invention, BDNF
genes and related nucleic acid sequences and subsequences,
including complementary sequences, may be used in
diagnostic hybridization assays. The BDNF nucleic acid
20 sequences, or subsequences thereof comprising about 15
nucleotides, can be used as hybridization probes.
Hybridization assays can be used to detect, prognose,
diagnose, or monitor conditions, disorders, or disease
states associated with changes in BDNF expression,
25 including, in particular, conditions resulting in sensory
neuron damage and degeneration of retinal neurons. Such
diseases and conditions include but are not limited to CNS
trauma, infarction, infection, degenerative nerve disease,
malignancy, or post-operative changes including but not
30 limited to Alzheimer's Disease, Parkinson's Disease,
Huntington's Chorea, and degenerative diseases of the
retina. For example, total RNA in a tissue sample from a
patient can be assayed for the presence of BDNF mRNA,
35 wherein the decrease in the amount of BDNF mRNA is

indicative of neuronal degeneration. Relatively high
levels of BDNF mRNA have been identified in neuroblastoma
tumor cells (see Section 14, infra, for details);
accordingly, in a specific embodiment of the invention,
detection of increased levels of mRNA using BDNF nucleic
acid probes can be utilized to diagnose neuroblastoma
tumor. The extremely low levels of BDNF synthesized by
most tissues render BDNF mRNA a particularly useful tumor
marker.

In alternate embodiments of the invention,
antibodies directed toward BDNF protein, peptide fragments,
or derivatives can be used to diagnose diseases and
disorders of the nervous system, including, in particular,
sensory disorders and degenerative diseases of the retina,
as well as those disorders and diseases listed supra. The
antibodies of the invention can be used, for example, in in
situ hybridization techniques using tissue samples obtained
from a patient in need of such evaluation. In a further
example, the antibodies of the invention can be used in
ELISA procedures to detect and/or measure amounts of BDNF
present in tissue or fluid samples; similarly, the
antibodies of the invention can be used in Western blot
analysis to detect and/or measure BDNF present in tissue or
fluid samples. An antibody of the invention which binds to
BDNF in ELISA and Western blots is described in Section 11,
infra.

In further embodiments of the invention, BDNF
protein, peptide fragments or derivatives can be used to
diagnose diseases and disorders of the nervous system. In
a particular embodiment and not by way of limitation,
labeled BDNF protein or peptide fragments can be used to
identify tissues or cells which express the BDNF receptor
in order to identify aberrancies of BDNF receptor
expression and consequently, potential abnormalities in the
tissue or cellular response to BDNF.

5.9.2. THERAPEUTIC APPLICATIONS

The present invention, which relates to nucleic acids encoding BDNF, and to proteins, peptide fragments, or derivatives produced therefrom, as well as to antibodies directed against BDNF protein, peptides, or derivatives,
5 may be utilized to treat diseases and disorders of the nervous system which may be associated with alterations in the pattern of BDNF expression or which may benefit from exposure to BDNF or anti-BDNF antibodies.

In various embodiments of the invention, BDNF
10 protein, peptide fragments or derivatives can be administered to patients in whom the nervous system has been damaged by trauma, surgery, ischemia, infection, metabolic disease, nutritional deficiency, malignancy, or toxic agents. The invention in particular can be used to
15 treat conditions in which damage has occurred to sensory neurons or retinal ganglion cells by administering effective therapeutic amounts of BDNF protein or peptide fragments or derivatives. In various specific embodiments of the invention, BDNF can be locally administered to
20 sensory neurons which have been severed, including, but not limited to, neurons in dorsal root ganglia or in any of the following tissues: the geniculate, petrosal, and nodose ganglia; the vestibuloacoustic complex of the VIIIth cranial nerve; the ventrolateral pole of the maxillo-
25 mandibular lobe of the trigeminal ganglion; and the mesencephalic trigeminal nucleus. It may be desirable to administer the BDNF-related peptides or BDNF protein by adsorption onto a membrane, e.g. a silastic membrane, that could be implanted in the proximity of the severed nerve.
30 The present invention can also be used for example in hastening the recovery of patients suffering from diabetic neuropathies, e.g. mononeuropathy multiplex. In further embodiments of the invention, BDNF protein or peptide
35 fragments or derivatives derived therefrom, can be used to

treat congenital conditions or neurodegenerative disorders,
including, but not limited to, Alzheimer's disease,
Parkinson's disease, Parkinson-Plus syndromes (in which
Parkinsonian symptoms result from degeneration of
dopaminergic neurons), such as Progressive Supranuclear

5   Palsy (Steele-Richardson-Olszewski Syndrome),
Olivopontocerebellar Atrophy (OPCA), Shy-Drager Syndrome
(multiple systems atrophy), and Guamanian Parkinsonism
dementia complex, and Huntington's chorea; in particular,
the invention can be used to treat congenital or

10 neurodegenerative disorders associated with sensory nerve
dysfunction and degenerative diseases of the retina. For
example, the BDNF protein, or peptide fragments, or
derivatives of the invention can be used in the treatment
of hereditary spastic paraplegia with retinal degeneration

15 (Kjellin and Barnard-Scholz syndromes), retinitis
pigmentosa, Stargardt disease, Usher syndrome (retinitis
pigmentosa with congenital hearing loss), and Refsum
syndrome (retinitis pigmentosa, hereditary hearing loss,
and polyneuropathy), to name but a few. It is possible

20 that a defect in BDNF synthesis or responsiveness may be
the underlying etiology for syndromes characterized by a
combination of retinal degeneration and other sensory
dysfunction.

            In a specific embodiment of the invention,
25 administration of BDNF protein, or peptide fragments or
derivatives derived therefrom, can be used in conjunction
with surgical implantation of tissue in the treatment of
Alzheimer's disease and/or Parkinson's disease. As
discussed in Sections 12 and 18, infra, BDNF may be used to
30 promote the survival of dopaminergic substantia nigra
neurons in a dose-dependent manner (Figure 34), supporting
the use of BDNF in the treatment of disorders of CNS
dopaminergic neurons, including, but not limited to,
Parkinson's disease [particularly in light of data
35

presented in Section 21, _infra_, which shows that BDNF may be used to prevent neurotoxicity caused by MPP, a toxin which is associated with the induction of a Parkinson's disease-like syndrome]. In addition, BDNF has been observed to sustain the survival of CNS cholinergic neurons

5 (Sections 12 and 16, _infra_) and, in particular, basal forebrain cholinergic neurons, indicating that BDNF may be useful in the treatment of disorders involving cholinergic neurons, including, but not limited to, Alzheimer's disease. It has been shown that approximately 35 per cent

10 of patients with Parkinson's disease suffer from Alzheimer-type dementia; BDNF produced according to the invention may prove to be useful single agent therapy for this disease complex. Similarly, BDNF produced according to the invention may be used therapeutically to treat

15 Alzheimer's disease in conjunction with Down's Syndrome. BDNF produced according to the invention can be used in the treatment of a variety of dementias as well as congenital learning disorders. It has also been found that BDNF appears to suppress the proliferation of astroglial cells,

20 supporting the use of BDNF for diminishing scar formation in the CNS (for example, following surgery, trauma, or infarct) as well as the use of BDNF in the treatment of astroglial derived CNS tumors. In yet another embodiment of the invention, BDNF may be used to upregulate the

25 expression of NGF receptor, and thereby may be advantageously administered prior to or concurrently with NGF to a patient in need of such treatment.

    As exemplified in Section 15, _infra_, administration of kainic acid may be used to induce an

30 increase in BDNF expression (and, to a lesser extent, NGF expression) in neurons, including hippocampal as well as cortical neurons. It has been observed (Section 15, _infra_) that inhibition of non-NMDA receptors blocked this kainic acid-mediated increase in BDNF expression. Accordingly,

35

expression of BDNF and BDNF related peptides of the invention may be induced _in_ _vitro_ or _in_ _vivo_ by the administration of kainic acid or related compounds or carbachol, histamine, or bradykinin, or related compounds thereof, which are found to have similar effects _in_ _vitro_

5 or _in_ _vivo_ or by agonists of non-NMDA glutamate receptors or other drugs which augment or mimic the action of acethycholine, histamine, or bradykinin. Induction of NGF expression may also be accomplished by administering kainic acid or related molecules or agonists of non-NMDA

10 receptors.

In further embodiments of the invention, BDNF protein, fragments or derivatives can be used in conjunction with other cytokines to achieve a desired neurotrophic effect. For example, and not by way of

15 limitation, according to the invention BDNF can be used together with NGF or with skeletal muscle extract to achieve a synergistic stimulatory effect on growth of sensory neurons wherein synergistic is construed to mean that the effect of the combination of BDNF protein, peptide

20 fragment, or derivative and a second agent achieves an effect greater than the same amount of either substance used individually. It is envisioned that BDNF may function synergistically with other CNS-derived peptide factors yet to be fully characterized, in the growth, development, and

25 survival of a wide array of neuronal subpopulations in the central nervous system.

It is further envisioned that, based on the full characterization of the BDNF molecule, novel peptide fragments, derivatives, or mutants of BDNF may be developed

30 which are capable of acting as antagonists of some, or all of the biological functions of BDNF. Such BDNF antagonists may be useful in the selective ablation of sensory neurons, for example, in the treatment of chronic pain syndromes.

35

In still further embodiments of the invention, antibodies directed toward BDNF protein, or peptide fragments or derivatives thereof, can be administered to patients suffering from a variety of neurologic disorders and diseases and who are in need of such treatment. For example, patients who suffer from excessive production of BDNF may be in need of such treatment. Anti-BDNF antibodies can be used in prevention of aberrant regeneration of sensory neurons (e.g. post-operatively), or, as discussed supra, in the treatment of chronic pain syndromes. In light of the high levels of BDNF mRNA found in neuroblastoma tissue, it is possible that BDNF serves as an autocrine tumor growth factor for neuroblastoma; accordingly, anti-BDNF antibodies can be administered therapeutically to achieve tumor regression in a specific embodiment of the invention.

5.10. PHARMACEUTICAL COMPOSITIONS

The active compositions of the invention, which may comprise all or portions of the BDNF gene product, including protein, peptide fragments or derivatives produced therefrom, or antibodies (or antibody fragments) directed toward BDNF protein, peptide fragments, or derivatives, or a combination of BDNF and a second agent, such as NGF or skeletal muscle extract, may be administered in any sterile biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water.

BDNF protein, peptide fragment or derivative may comprise an amino acid sequence or subsequence thereof substantially as depicted in Figure 1 or Figure 5; it may be preferable to use BDNF protein comprising, in particular, all or a portion of the amino acid sequence from about amino acid 134 to about amino acid 252, as depicted in Figure 1, or a functionally equivalent

sequence, as this subsequence is believed to comprise the functional portion of the BDNF molecule. BDNF may be derived from sequences corresponding to the BDNF genes of any suitable species, including, but not limited to, human, pig, rat, chicken, cow, dog, sheep, goat, cat, rabbit, etc.

5 The amount of BDNF protein, peptide fragment, derivative, or antibody which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. Where

10 possible, it is desirable to determine the dose-response curve and the pharmaceutical compositions of the invention first in vitro, e.g. in the BDNF bioassay systems described supra, and then in useful animal model systems prior to testing in humans. Based on in vitro data, in a specific

15 embodiment of the invention, a pharmaceutical composition effective in promoting the survival of sensory neurons may provide a local BDNF protein concentration of between about 5 and 25 ng/ml, and, preferably, between 10 and 20 ng/ml of BDNF. In an additional specific embodiment of the

20 invention, a pharmaceutical composition effective in promoting the growth and survival of dopaminergic or cholinergic neurons may provide a local BDNF protein concentration of between about 10 ng/ml and 100 ng/ml.

  Methods of introduction include but are not

25 limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, and intranasal. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including

30 intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

35

Further, it may be desirable to administer the
pharmaceutical compositions of the invention locally to the
area in need of treatment; this may be achieved by, for
example, and not by way of limitation, local infusion
during surgery, by injection, by means of a catheter, or by
5   means of an implant, said implant being of a porous, non-
porous, or gelatinous material, including membranes, such
as sialastic membranes, or fibers.

The invention also provides for pharmaceutical
compositions comprising BDNF proteins, peptide fragments,
10 or derivatives administered via liposomes, microparticles,
or microcapsules. In various embodiments of the invention,
it may be useful to use such compositions to achieve
sustained release of BDNF and BDNF-related products.

It is envisioned that it may be possible to
15 introduce cells actively producing BDNF, BDNF related
substances, BDNF antagonists, or anti-BDNF antibodies into
areas in need of increased or decreased concentrations of
BDNF.

20   6.   EXAMPLE: MOLECULAR CLONING AND CHARACTERIZATION
          OF PORCINE BRAIN DERIVED NEUROTROPHIC FACTOR cDNA
The extreme rarity of BDNF protein in tissues
precluded the use of standard strategies toward cloning the
BDNF gene. Instead, a limited amount of protein sequence
25 data was expanded, utilizing DNA amplification technology,
as follows:
        (i)   Microgram quantities of BDNF were purified from
               kilogram quantities of porcine brain.
        (ii)   Purified BDNF was analyzed by protein
30   microsequencing techniques, and the amino acid
               sequence of a stretch of 36 amino acid residues
               was determined.
        (iii) Based on the amino acid sequence determined in
               (ii), oligonucleotides were synthesized and then
35

used as primers in polymerase chain reaction,
using porcine superior colliculus cDNA as
template in order to amplify DNA encoding the
defined amino acid fragment.

(iv)   The DNA reaction product of (iii) was sequenced,
       and

(v)   Corresponding oligonucleotide primers were
       synthesized and utilized in polymerase chain
       reaction with porcine superior colliculus cDNA
       to generate overlapping fragments of DNA
       representing BDNF mRNA upstream as well as
       downstream of sequences encoding the original 36
       amino acid fragment. Thus, the complete coding
       region for porcine BDNF was molecularly cloned
       in two overlapping fragments.

The details of each of these steps, as well as further
characterization of the BDNF gene, are set forth below.

6.1. MATERIALS AND METHODS
6.1.1. PURIFICATION OF BDNF FROM PORCINE BRAIN

       Purification of BDNF from porcine brain was
performed by the procedure essentially as set forth in
Hofer and Barde (1988, Nature 331:261-262).

       Six kg of pig brain was homogenized with an
Ultra-Turrax homogenizer in 0.2 M sodium phosphate buffer,
pH 6.0, containing 1 mM EDTA and freshly added
phenylmethanesulphonyl fluoride (1 mM) at a ratio of 1 kg
of brain to 2 liters of buffer. The pH of the supernatant
was brought to pH 4.0 with 1 N HCl and stirred for 2 hr at
4°C. After centrifugation for 25 min at 20,000 x g the
combined supernatants (adjusted to pH 6.0 with 1 N NaOH),
corresponding to 3 kg of brain, were stirred for 2 hr with
1 liter of preswollen carboxy-methyl-cellulose which had
been equilibrated with 0.1 M sodium phosphate, pH 6.0.
After two washes with a total of 20 liter of 0.1 M sodium

phosphate, pH 6.0, the slurry was poured into a column and washed overnight in the same buffer containing 0.13 M NaCl. Active fractions were eluted with phosphate buffer containing 0.5 M NaCl and subsequently dialyzed against 2 x 5 liter of 5 mM potassium phosphate, pH 6.8. The dialyzed

5 fractions resulting from processing 2 x 3 kg of starting material were applied to a 130 ml bed volume hydroxyapatite column which had been previously equilibrated with 5 mM potassium phosphate, pH 6.8. The column was then eluted with a linear gradient composed of 500 ml of 5 mM and 500

10 ml of 700 mM potassium phosphate, both pH 6.8, whereby the BDNF activity was eluted at approximately 500 mM potassium phosphate (see Barde et al., 1982, EMBO J. 1:549-553). By addition of 1.0 M potassium phosphate, the pooled active fractions were adjusted to a final molarity of 700 mM

15 potassium phosphate and applied to a 5 ml phenyl-sepharose column equilibrated with 700 mM potassium phosphate, pH 6.8. After washing with 40 ml of the same buffer, BDNF activity was eluted with 0.1 M potassium phosphate, pH 6.8 dialyzed against distilled water, and lyophilized. The

20 lyophilized material was taken up in an SDS-gel electrophoresis sample buffer containing 0.1% SDS and no mercaptoethanol as described in Barde et al. (1982, EMBO J., 1:549-553), before being applied to an SDS-gel comprised of a linear (rather than exponential) gradient of

25 10-25% acrylamide. After completion of electrophoretic separation, the gel was briefly stained (10 min) with Coomassie blue, destained for 20 min, and a band migrating at the level of cytochrome c was cut out and electrophoretically eluted from the gel. SDS was removed

30 as described Barde et al. (1982, EMBO J., 1:549-553). The purification procedure that eventually led to the microsequencing of BDNF was modified (according to Hofer and Barde, 1988, Nature 331:261-262) and does not make use

35 of gel electrophoresis as the last step of purification.

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6.1.2. PROTEIN SEQUENCING

BDNF was either sequenced directly (55 pmole as determined by amino acid analysis, with an initial yield of 40 pmole for the amino terminal histidine) or cleaved as follows: 5 to 10 μg BDNF (from 5 different preparations)

5 were cleaved according to the following procedures. V8:1μg S. aureus V8 (Miles) was added to 5 μg BDNF in 0.2 M NH$_4$CO$_3$, pH 8.0 containing 10% acetonitrile (total volume: 50 μl) and incubated overnight at room temperature. Trypsin: 1 μg TPCK-treated trypsin (bovine pancreas, Sigma

10 Type XIII) was added to 8 μg BDNF in Tris-HCl (0.1 M, pH 8.0) containing 10 mM CaCl$_2$ (total volume: 40 μl) and incubated overnight at 37°C. Cyanogen bromide (CNBr): 10 μg BDNF were incubated for 3 hours at room temperature (total volume: 60 μl) with 10% (w/v) CNBr in 70% (v/v) formic acid

15 (final concentrations). After addition of 500 μl H$_2$O at the end of the reaction, the sample was concentrated to 50 μl in a speed-vac. 50 μl Tris-HCl (1.0 M, pH 8.0) were added together with 5 μl β-mercaptoethanol and the sample was incubated overnight at 37° C. After addition of 5 μl

20 iodomethane, the sample was evaporated to dryness on a speed-vac. Reduction and alkylation of BDNF after CNBr cleavage were found to be necessary: no fragments were obtained without reduction, as revealed by HPLC and Swank-Munkres SDS-gel electrophoresis (Swank and Munkres, 1971,

25 Anal. Biochem. 39:462-477). This indicates that disulfide bridges are present in BDNF, and arranged in such a way that none of the cleavages can give rise to free peptides. After all cleavages, the dried samples were resuspended in 0.1% trifluoroacetic acid (TFA) and applied onto a reverse

30 phase C8 microbore HPLC column (Applied Biosystems) and the peptides eluted at 0.1 ml/min, using a 60 min linear gradient of 0-60% acetonitrile in 0.1% TFA. Detection was at 214 nm with a Waters 441 UV detector. The peptides were sequenced by automated Edman degradation on a gas phase

35

microsequencer (model 470A Applied Biosystems), according to Hewick (1981, J. Biol. Chem. <u>256</u>:7990-7997) and Hunkapillar (1983, Methods Enzymol. <u>91</u>:227-236). Detection of the PTH amino acids was as described by Lottspeich (1985, J. Chromatogr. <u>326</u>:321-327).

6.1.3. PREPARATION OF DNA TEMPLATES

Pig genomic DNA was isolated according to the method of Herrmann and Frischauf (1987, Methods Enzymol. <u>152</u>:180-183).

For preparation of cDNA, total RNA was obtained from a 6 gram sample of superior colliculus of pig brain. The tissue specimen was obtained, dissected and frozen in liquid nitrogen at a local slaughterhouse. Standard methods (Okayama et al., 1987, Methods Enzymol. <u>154</u>:3-28) were utilized to extract the RNA. Eighty μg total RNA was transcribed with the reverse transcriptase of the Moloney murine leukemia virus (BRL, according to the manufacturer's instructions except for the addition of 1 μl RNasin and the omission of actinomycin D), using the primer mixture CGGATCCGAATTCTGCAGTTTTTTTTTTTTT with A, C or G as terminal 3' nucleotide (oligo3, designed to match a 3' poly-A stretch and containing recognition sites for <u>BamHI</u>, <u>EcoRiI</u> and PstI).

6.1.4. POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) was performed according to the method described in Saiki et al., (1985, Science <u>230</u>:1350-1354).

6.2. RESULTS AND DISCUSSION
6.2.1. RESULTS OF PROTEIN MICROSEQUENCING

The results of protein microsequencing are presented in Table IV.

TABLE IV

EXPERIMENTALLY DETERMINED PEPTIDE SEQUENCE OF BDNF

N-terminal	HSDPARRGELSV
V8	XVTAADKKTAVD
V8	KVPVSKGQLKQYFYE
CNBr	XGGTVTVLEKVP(V)(S)
CNBr	GYTKEGXRGIXRGI
Trypsin	(T)AVDMSGGTVTVLEK
Trypsin	ALTMDSK

Combined sequence VTAADKKTAVDMSGGTVTVLEKVPVSKGQLKQYFYE
underlined amino acids represent sequences encoded by
oligonucleotide primers used in PCR; see Section 6.1.2.

Four contiguous segments of amino acid sequences were
determined using Edman degradation, which could, in turn,
be used to deduce a sequence of 36 amino acids representing
approximately one-third of the entire amino acid sequence.

6.2.2.   SYNTHESIS OF OLIGONUCLEOTIDES AND USE OF PCR TO OBTAIN DNA ENCODING AN AMINO ACID FRAGMENT

Two fully degenerate 17-mer oligonucleotide
primers were chemically synthesized, based on the coding
sequences for segments of six amino acids near the amino-
terminal and carboxyl-terminal ends, respectively, of the
36 amino acid residue fragment described in Section 6.2.1.,
supra. In particular, two separate mixtures of 17-mer
oligonucleotides (corresponding to all possible codons and
designated oligo1 and oligo2) based on the sequence AADKKT
(sense) and KQYFYE (antisense) (underlined in Table III)
were chemically synthesized, and 150 pmole of each primer
were added to 1 μg pig genomic DNA template. Thirty-Five

amplification cycles were performed using the polymerase
chain reaction (PCR) according to the instructions of the
manufacturers (GeneAmp^{TM}, Perkin-Elmer Cetus).
Denaturation was at 94°C for one minute, primer annealing
at 45°C for 2 min. and primer extension at 72°C for 2 min.
5   A DNA band of the predicted size (101 bp) was cut out from
a 3% agarose gel (stained with ethidium bromide) and
asymmetrically amplified as described by Innis et al.
(1988, Proc. Natl. Acad. Sci. U.S.A. 85:9436-9440) with a
100-fold excess of either oligol or oligo2.
10

6.2.3.   NUCLEOTIDE SEQUENCE OF cDNA FRAGMENT
         The resulting sense and antisense DNA fragments
were sequenced by the dideoxynucleotide chain termination
method (Sanger et al., 1979, Proc. Natl. Acad. Sci. U.S.A.
15   72:3918-3921) using the ^{32}P end-labelled oligonucleotides 1
and 2 as primers. The observed nucleotide sequence
contained only one open reading frame uninterrupted by a
chain termination codon. The deduced amino acid sequence
for this open reading frame was completely consistent with
20   the actual amino acid sequence determined for this region
of porcine BDNF.

6.2.4.   CLONING OF THE ENTIRE PORCINE BDNF cDNA
         The complete coding region for porcine BDNF was
25   molecularly cloned in two overlapping segments. In order
to obtain the 3' portion (relative to the sense strand) of
the BDNF cDNA, a 30-mer oligonucleotide primer containing
21 bases of exact sense strand BDNF sequence, from within
the region described above, was synthesized to serve as a
30   "sense primer". The nucleotide sequence of this primer was
oligo 4,   (5')AAACTAGTCGACGGCAGTGGACATGTCGGG(3')
[underlining indicates the sequence corresponding to sense
strand of BDNF, from positions 643 to 663 on Figure 1,
encoding the amino acid sequence Thr-Ala-Val-Asp-Met-Ser-
35

Gly (first two bases of Gly codon)]. The additional 9
nucleotides at the 5' end of this primer were included to
provide convenient restriction endonuclease cleavage sites
for Spe I and Sal I for use in molecular cloning. A
three-fold degenerate 31-mer oligonucleotide primer was
5 designed to be complementary to a stretch of poly-A
preceded by any nucleotide (T, G, or C) on the sense strand
of cDNA, and to contain recognition sites for the
restriction endonucleases BamHI, EcoRI, and PstI. The
sequence of this "antisense" primer (oligo 3) was
10 (5')CGGATCCGAATTCTGCAGTTTTTTTTTTTTX(3'), where X=A, C, or
G. The synthetic oligonucleotide primers were utilized to
amplify sequences from the porcine superior colliculus cDNA
preparation described above, by PCR. Specifically, the 3'
amplified DNA was obtained by using 10 μl of the reverse
15 transcription reaction, 150 pmole of the sense primer
(oligo 4) and 150 pmole of oligo3 as antisense primer in a
PCR reaction. A Southern blot analysis was performed on
the amplified DNA products and the band giving a
hybridization signal with the ^{32}P-end-labelled
20 oligonucleotide AAGGATCCTGCAGTTGGCCTTTCGAGACGG (oligo5,
used as antisense primer in the 5' reaction described below
and containing recognition sequences for BamHI and PstI)
was cut out, extracted by the glassmilk method (gene
clean™, digested with EcoRI and SalI, cloned into a
25 Bluescript SK^{+} plasmid (Stratagene) and sequenced.
        In order to obtain the remainder (upstream or 5'
portion) of the BDNF coding sequence, cDNA was prepared as
described above, and poly-A tails were added at the 5' ends
using terminal deoxynucleotide transferase. The same
30 mixture of three 31-mer oligonucleotides each containing a
stretch of 12 consecutive T residues, described above, was
used to give a primer complementary to these added poly-A
tails. A unique 30-mer oligonucleotide primer containing
17 bases corresponding to the complementary strand of the
35

BDNF coding sequence, and with recognition sites for
restriction endonucleases BamHI and PstI was synthesized.
The sequence of this primer was
(5')AAGGATCCTGCAGTTGGCCTTTCGAGACGG(3') (oligo5, supra;
underlining indicates the region complementary to the sense
5 strand of the coding sequence of BDNF, from positions 709
to 693 on Figure 1); this corresponds to the segment
encoding the amino acid sequence Pro(last two bases of
codon)-Val-Ser-Lys-Gly-Gln. The primers were added to the
poly-A-tailed cDNA, and amplification by PCR was carried
10 out as above. The reaction products were cut with PstI and
cloned into Bluescript vector and the nucleotide sequence
was determined by the dideoxynucleotide chain termination
method.

15   6.2.5.   NUCLEOTIDE SEQUENCE OF PORCINE BDNF cDNA
            The combined nucleotide sequence determined from
the overlapping porcine BDNF cDNA clones is shown in Figure
1 together with the deduced amino acid sequence. The
sequence comprises an open reading frame for a polypeptide
20 of 252 amino acids. The identification of the initiator
Met codon (ATG) is based on the presence of two adjacent
chain termination codons (TAG-TGA) in the same reading
frame, beginning 36 base-pairs upstream. The amino
terminus of porcine BDNF, determined by direct sequence
25 analysis on purified protein corresponds to residue His134
of this polypeptide. Thus, the sequencing data show that
mature BDNF is derived by processing from a precursor
polypeptide. The residue His134 is preceded immediately by
the sequence Arg-Val-Arg-Arg. Such sequences of one basic
30 amino acid residue followed by a neutral residue and then
two more basic residues have been implicated as target
sites for proteolytic processing of precursor polypeptides.
The deduced amino acid sequence of mature BDNF predicts a
35 protein of 119 amino acids (molecular weight 13,511

daltons) with basic charge (pI = 9.99), properties
consistent with previous characterization of BDNF by
evaluation of biologically active factor after
fractionation by two-dimensional gel electrophoresis. The
amino acid sequence of portions of BDNF determined by
5 protein microsequencing (a total of 64 amino acid residues)
is in complete agreement with the amino acid sequence
deduced from the nucleotide sequence of cDNA clones
(underlined in Figure 1). The sequence of the precursor
polypeptide is consistent with processing of BDNF in at
10 least two steps: first, a signal peptide of perhaps 18
residues would be cleaved from the amino terminus, followed
by cleavage between Arg133 and His134 to liberate the
mature polypeptide. If this model is correct, then the
precursor could be designated preproBDNF.
15

7.   EXAMPLE: BDNF GENE IS DISTINCT FROM THE
     NGF GENE IN DIVERSE VERTEBRATE SPECIES
            7.1.   MATERIALS AND METHODS
      7.1.1.   PREPARATION OF NGF AND BDNF DNA PROBES
20   A plasmid containing a synthetic gene encoding
mature human NGF, incorporating the normal human NGF coding
sequence with a few conservative codon substitutions to
introduce convenient restriction endonuclease cleavage
sites, was purchased from British Biotechnologies Limited.
25 A pair of 18-mer oligonucleotide primers were synthesized
to permit amplification of a 270 base pair segment of this
gene, corresponding to the coding region for amino acid
residues 9 to 111, by PCR. In order to obtain a labeled
DNA probe, a PCR reaction of 10 cycles was carried out with
30 ^{32}P-dCTP. A BDNF probe was obtained by similar procedures,
except that amplification was carried out using porcine
genomic DNA as the original template, and the segment
amplified corresponded to the coding region for amino acids
28 to 111 of mature BDNF. A complementary strand
35

("antisense") primer corresponding to the region of amino acids 106 to 111 was synthesized, and had the sequence (5')ACATACACAGGAAGTGTC(3'). A sense strand oligonucleotide primer was prepared for the region of amino acid residues 28-33 [(5')GCAGTGGACATGTCGGGT(3')]. Southern blot

5 hybridization (Southern, 1975, J. Mol. Biol. 98:503-517) with these two probes was carried out under stringent conditions in 2 x SSC at 68°C.

7.1.2. SEQUENCING OF BDNF GENES FROM VARIOUS SPECIES

10   The same two 18-mer oligonucleotides bracketing the coding region for amino acids 28 to 111 of mature porcine BDNF, described above, were used as primers to amplify, under standard PCR conditions, 252 base pair segments of genomic DNA of pig, rat, chicken, and human,

15 and the resulting DNA reaction products were sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1979, Proc. Natl. Acad. Sci. U.S.A. 72:3918-3921). In some cases the band of amplified DNA was cut out and extracted after agarose gel electrophoresis, and

20 reamplified prior to sequencing. In other cases reamplification was not essential.

7.2. RESULTS AND DISCUSSION

       Prior to the instant invention, BDNF protein had
25 been purified only from pig. It was of paramount importance to demonstrate unequivocally that BDNF is not simply the porcine nerve growth factor beta subunit (beta-NGF, or simply referred to herein as NGF), the purification and molecular cloning of which has not been reported to

30 date. This was especially critical because the previously reported physical properties of porcine BDNF are virtually identical to those of the β-NGF monomer from a variety of species. The lack of a neutralizing antibody against

35 porcine BDNF, and the previous lack of amino acid or

nucleotide sequence information on porcine BDNF made it
impossible to determine the exact relationship between BDNF
and NGF. It was conceivable that the observed differences
in biological activity between BDNF and NGF might, for
example, simply reflect differences in NGF between pig and
5   certain other species (e.g. mouse), or might result from
differential modification of the NGF protein molecule in
different tissues (e.g. pig brain versus mouse salivary
gland), or from modifications inadvertently introduced into
the protein at some step(s) during purification from pig
10   brain.

If BDNF were found to be clearly distinct from
NGF, it was of great interest to determine whether the BDNF
gene is present in other species, notably man. Prior to
the instant invention, no information was available
15   concerning this point, because BDNF was purified from only
one species. The presence of neurotrophic activity
apparently distinct from NGF in a variety of crude extracts
and conditioned media did not imply the existence of a
substance identical or substantially equivalent to porcine
20   BDNF.

Comparison of the predicted amino acid sequence
of porcine BDNF with the known sequences of NGF from a
number of species (human, bovine, guinea pig, mouse,
chicken, and snake) indicated that BDNF is significantly
25   less closely related to any vertebrate NGF than the NGF's
are to each other (Figure 2). A striking feature of the
primary structure of mature BDNF is its similarity to that
of NGF; with only 3 gaps introduced in the NGF sequences to
optimize matching, 51 identities are common to the various
30   NGFs (from snake to man) and BDNF (Figure 2). Importantly,
those identities include all 6 cysteine residues. Though
the exact arrangement of BDNF's disulfide bridges are not
yet known, it is clear that such bridges are present (Table
III, legend). The 3 tryptophan and 2 phenylalanine
35

residues found in BDNF are found at identical positions in
NGF. We also note that 6 aspartic acid residues (out of 7
in BDNF) and 7 valine (out of 9) are present at identical
positions in mammalian NGFs and BDNF. These 5 amino acids
account for about half of the amino acid identities between
5 the 2 proteins. Conversely, there are some striking
differences between BDNF and all NGFs: in addition to the
3 gaps already mentioned, there are also 21 positions at
which the amino acids are identical in all NGFs, but
different in BDNF.

10   Most of BDNF's precursor sequence is unrelated
to that of NGF, with 2 exceptions: the putative secretory
signal sequence of BDNF shows 5 amino acid identities (out
of 18 amino acids) and an overall striking relatedness with
the signal sequence of mouse NGF, where cleavage has been
15 demonstrated to occur after an alanine residue found in
position 18 after the translation initiation methionine
(Edwards et al., 1988, Mol. Cell Biol. 8:2456-2464). It
seems likely that the alanine also found in position 18 in
BDNF represents a potential cleavage site for the removal
20 of BDNF's signal sequence. The other similarity with NGF
starts at the only N-glycosylation consensus sequence
(double underlined in Fig. 1), corresponding to asparagine
126. This asparagine is located 8 amino acids before the
cleavage site giving rise to mature BDNF. The same
25 arrangement is found in several NGFs, as well as the
sequence Arg-X-Basic-Arg as the last 4 amino acids of the
precursor (Schwarz et al., 1989, J. Neurochem. 52:1203-
1209)

30   Proof that NGF and BDNF are encoded by distinct
genes in a variety of vertebrate species was obtained by
preparing DNA probes from molecularly cloned human NGF and
porcine BDNF, and carrying out Southern blot hybridization
with with genomic DNA digested with restriction
35 endonuclease EcoRI. Genomic DNAs were analyzed from the

following sources: human, monkey, rat, mouse, dog, bovine,
rabbit, chicken, and yeast. DNA was digested with EcoRI,
and analyzed by Southern blotting, on duplicate filters,
with a ^{32}P-labeled human NGF probe and a porcine BDNF
probe. Single bands were observed with each probe in all
5   organisms tested except yeast. In most cases the bands
hybridizing with the NGF and BDNF probes in any one
organism were of different electrophoretic mobility,
although in some cases (e.g. mouse DNA), the EcoRI
fragments hybridizing to NGF and BDNF probes were of
10   approximately the same size, and could not be resolved
under the electrophoretic conditions used (Figure 3).

A portion of the coding sequences for mature
BDNF was amplified by PCR from genomic DNA of pig, rat,
chicken, and human, and the nucleotide sequences
15   determined. DNA sequence analysis of the amplified region
from porcine genomic DNA exactly confirmed the sequence
results obtained with molecular clones from porcine brain
cDNA. The genomic sequences of rat, human, and chicken
BDNF for this 252 base pair segment were also determined
20   (Figure 5). Remarkably, in rat and human the deduced amino
acid sequence for the region of at least amino acids 28 to
111 was identical to that of porcine BDNF, although a
number of nucleotide differences (e.g. conservative changes
in the third position of a codon) were observed among the
25   various species. In chicken a single amino acid
substitution was observed in this region; residue 61 of the
mature protein is a lysine in chicken compared to a
methionine in mammalian BDNFs. The sequence data, together
with the Southern blot hybridization experiment described
30   above, provided unequivocal proof that BDNF is encoded by a
highly conserved gene distinct from that encoding NGF.

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8.   EXAMPLE: EXPRESSION OF BDNF RNA IN NEURONAL
VERSUS NON-NEURONAL TISSUES

8.1.   MATERIALS AND METHODS

8.1.1.   PREPARATION OF RNA

Total RNA was extracted from adult female mice
tissues according to Okayama et al. (1987, Methods Enzymol.
154:3-28). Briefly, frozen tissue was homogenized in 5.5 M
guanidinium thiocyanate, the lysate centrifuged to remove
debris, and the supernatant overlaid onto a cushion of
cesium trifluoroacetate adjusted to a density of 1.51 g/ml.
After a 24 hour centrifugation at 125,000 x g in a SW 27
swinging bucket rotor (Beckmann), the RNA was resuspended
and precipitated with ethanol and 8 M ammonium acetate and
stored at -70°C. Electrophoresis was according to Lehrach
et al. (1977, Biochemistry 16:4743-4751) on 1.3% agarose-
formaldehyde gels. The RNA was transferred to nylon
membranes (Hybond-N, Amersham) and hybridized overnight at
62°C in 1 ml 2 X SSC containing 50% formamide with a ^{32}P-
cRNA mouse BDNF probe (10^7 cpm, see below). Washing was
for 60 min at 65°C in 0.1 X SSC. After washing , the blot
was incubated for 60 min at room temperature with 0.1 μg/ml
RNAse A (Pharmacia) and the film exposed at -70°C (with
intensifying screen) for 48 hours.

8.1.2.   PREPARATION OF cRNA PROBE

A mouse brain cDNA library was screened with 2
independent BDNF oligonucleotides. Double positive clones
were isolated and subcloned into the EcoRI site of a
Bluescript SK$^+$ plasmid (Stratagene). The nucleotide
sequence corresponding to nucleotides 350 to 829 of the pig
sequence (see Fig. 1) was determined. In this sequence, a
total of only 4 amino acid differences were found between
mouse and pig BDNF, indicating a remarkable degree of
conservation of this domain between pig and mouse BDNF. A
single stranded RNA probe was prepared using this template

and the T3 polymerase (Promega). The specific activity of
this probe was 10^8 cpm/μg.

8.2. RESULTS AND DISCUSSION

Northern blot analysis was used to evaluate the
expression of BDNF mRNA in neuronal versus non-neuronal
tissues. Northern blot analyses were performed using mouse
tissues, allowing more rapid processing for RNA extraction
than pig tissues. A ^{32}P-cRNA probe detected a signal at
about 1.45 kb in the brain (Fig. 4) and spinal cord (data
not shown). Significantly, no signal was detected in any
other tissue including kidney, gut, lung, liver, spleen,
heart and muscle (Fig. 4). Assuming the size of the pig
mRNA to be similar to that of the mouse, the cDNA sequence
shown in Figure 2 represents over 80% of the complete mRNA
sequence.

A significant observation in the delineation of
BDNF's physiology is that the mRNA coding for this protein
was only found in the central nervous system and not in 7
non-CNS tissues. BDNF mRNA was found not only in total
brain (Fig. 4), but also in the spinal cord and the
superior colliculus (the sequence presented in Figure 1
derives entirely from a superior colliculus cDNA template).
These data support the idea that BDNF is a target-derived
neurotrophic factor and that BDNF-responsive neurons are
either intrinsic to the CNS or directly connected with CNS
structures. Indeed, all the neurons known to respond to
BDNF so far either project into the CNS, like the dorsal
root or the cranial sensory ganglia (Lindsay et al., 1985,
Dev. Biol. 112:319-328; Davies et al., 1986, J. Neurosci.
6:1897-1904), or are CNS neurons like the retinal ganglion
cells (Johnson et al., 1986, J. Neurosci. 6:3031-3038).
Clearly, detailed studies need to be done to examine the
exact distribution of the sites of synthesis of BDNF in the
CNS, but it is already evident that the distribution of

BDNF mRNA is very different from that NGF mRNA, found in many non-CNS tissues (Heumann et al., 1984, EMBO J. 3:3183-3189; Shelton et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:7951-7955).

5   9.   EXAMPLE: MOLECULAR CLONING AND CHARACTERIZATION
           OF HUMAN AND RAT BDNF GENES

                    9.1.   MATERIALS AND METHODS
                9.1.1. GENOMIC DNA AND cDNA LIBRARIES
                    An adult human retina cDNA library, in lambda-
10   ZAPII was obtained from Stratagene. A human placenta
     genomic DNA library in EMBL3/SP6/T7 was obtained from
     Clontech. A human fetal brain cDNA library in λgt11 was
     obtained from Clontech. A rat genomic DNA library in
     EMBL3/SP6/T7 was obtained from Clontech. Both genomic
15   libraries were prepared by partial digestion of genomic DNA
     with Sau 3A restriction endonuclease and ligation into the
     BamHI site of the vector. A rat brain cDNA library in
     lambda-ZAPII was obtained from Stratagene.

20   9.1.2.   PREPARATION OF BDNF DNA PROBES
                 ^{32}P-labeled BDNF DNA probes were prepared using
     the same oligonucleotide primers described in Section
     7.1.1., supra in PCR reaction with human genomic DNA in
     order to amplify the coding region for residues 28-111 of
25   human BDNF. In parallel, a rat BDNF-specific ^{32}P-labeled
     probe was obtained, using rat genomic DNA as a template for
     PCR.

                 9.1.3.   SCREENING OF LIBRARIES
30   Lambda phage libraries were screened according
     to standard methods (Benton and Davis, 1977, Science
     196:180-182; Maniatis et al., 1978, Cell. 15:687-701),
     hybridizing in 50% formaldehyde with dextran sulfate and
     Denhardt's solution at 42°C. Filters were prehybridized at
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42°C in 50% formamide, 5XSSCPE, 10% Denhardt's, 0.5 mg/ml
Salmon sperm DNA, 0.1% SDS, and 10% dextran sulfate.
Hybridization was carried out in the same buffer except
that Denhardt's solution was at 2%, Salmon sperm DNA was at
0.1 mg/ml, and SDS and dextran sulfate were omitted. After
5   hybridization filters were washed at 68°C. Human BDNF
probe was used to screen human genomic DNA and retina cDNA
libraries; rat BDNF probe was used to screen rat genomic
DNA and brain cDNA libraries. Libraries were also screened
with human and rat NGF probes, prepared as described in
10   Section 7.1.1. supra.

9.2. RESULTS AND DISCUSSION

At least 670,000 plaques were screened from each
library. Positive human clones were considered to be those
15   which hybridized with the human BDNF probe, but not with
the human NGF probe described above. BDNF genomic clones
were obtained from both the human and rat libraries at a
frequency consistent with representation of the BDNF gene
at one copy per haploid genome. For both rat and human
20   genomic libraries, approximately one million plaques were
screened. Three positives were obtained from the rat
genomic library and one was obtained from the human genomic
library. Positive clones from the human retinal and rat
brain cDNA libraries were obtained at a frequency
25   consistent with a gene expressed at very low levels; in rat
brain cDNA, 2 positives were identified in 670,000; in
human retinal cDNA library, one in 670,000 clones were
identified. No positive clones were detected among 670,000
from the commercial cDNA library prepared from human fetal
30   brain. Nucleotide sequencing was carried out on human BDNF
cDNA and genomic clones, using synthetic oligonucleotide
primers representing exact sequences from within the human
and rat BDNF coding region, as determined in Section
7.1.2., supra. The longest human cDNA clone obtained had
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an insert of approximately 1.6 to 1.8 kbp, and, as expected, contained the exact sequence of the portion of human BDNF determined after direct amplification from human genomic DNA, as described in Section 7.2., supra.
Detailed sequence analysis of this cDNA (Figure 5) clone

5   revealed an open reading frame encoding a polypeptide of 247 amino acids, similar but not identical to the full-length precursor to porcine BDNF. Within the region corresponding to the mature BDNF polypeptide (e.g. from the codon for His134 to the chain termination codon) no

10   differences in deduced amino acid sequence were found; all nucleotide substitutions between pig and man were conservative with respect to coding specificity. The remainder of the putative BDNF precursor polypeptide showed some amino acid sequence differences between human and pig,

15   most notably the absence in the human BDNF gene of 5 of 6 consecutive Ser codons seen in pig, resulting in a slightly shorter polypeptide (247 versus 252 amino acids).

     Libraries prepared in vector EMBL3 have inserts of between 10 to 23 kbp of foreign DNA. The exact insert

20   size in the human genomic BDNF clone analyzed in detail was not determined. However, the clone contained a single EcoRI restriction endonuclease fragment of approximately 4 kbp that hybridized with the labeled BDNF probe used to screen the library. This fragment is the expected size,

25   based on the results of Southern blot hybridization of human genomic DNA with the porcine BDNF probe described above. Sequence analysis was carried out on the human clone, using synthetic oligonucleotides representing cDNA sequence to prime DNA synthesis from the bacteriophage DNA

30   template. The sequence of the coding sequence for the putative human BDNF precursor was found to be identical to that in the human cDNA clone, with the exception of a single nucleotide substitution in the prepro region,

35   corresponding to an amino acid substitution of methionine

(ATG) for Valine (GTG), at nucleotide 785, in Figure 5.
This change may reflect a polymorphism in the human genome.
As in the case of the human NGF gene, no intervening
sequences were detected within the coding sequence for the
putative human preproBDNF. Rat cDNA sequence data is also
5   presented in Figure 5.

10.   EXAMPLE: EXPRESSION OF RECOMBINANT BDNF
10.1.   MATERIALS AND METHODS
10.1.1.   PREPARATION OF A BDNF EXPRESSION VECTOR

10   The sequence corresponding to pig preproBDNF was
obtained by using the oligonucleotide primers (150 pmole
each) ATAATCTAGATGACCATCCTTTTCCTT (sense)
ATAATCTAGACTATCTTCCCCTCTTAAT (antisense) in a PCR reaction
using 1 μg of pig genomic template (each primer has an
15 added XbaI site). The amplification reaction was as
described, except that the annealing temperature was 50° C.
After digestion with XbaI, the amplified DNA was ligated in
the XbaI site of the plasmid pCMV^1 to produce pCMV^1-pBDNF^{-1}
(-1 denotes sense orientation in Figure 6 and corresponds
20 to COS+ in Table V), and the plasmid was introduced into
XL-1 bacteria by electroporation.

10.1.2.   EXPRESSION OF BDNF IN COS CELLS
        pCMV1-pBDNF plasmid DNA from positive clones
25 (checked by hybridization with oligo5, Fig. 2) was cut with
both XbaI and PstI. The size of the resulting products
allowed us to determine the orientation of the inserts, and
both plasmids were used to transfect COS cells by the
calcium phosphate method (Chen et al., 1987, Mol. Cell
30 Biol. 7: 2745-2752), and the growth medium collected after
24 h. BDNF activity was tested in the chick embryo dorsal
root ganglion bioassay discussed in detail supra.

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10.2. RESULTS AND DISCUSSION

E8 chick spinal sensory neurons were plated (6,000 per well), incubated for 24 h and counted after 24 h (Lindsay et al., 1985, Develop. Biol. 112: 319-328). The values are the means of triplicate determinations ± standard deviation. BDNF and NGF were used at 1 ng/ml, concentrations at which maximal survival is observed with either factor. COS+ refers to cells transfected with plasmids containing the BDNF insert in the sense orientation, COS- to cells transfected with plasmids containing the BDNF inserts in the reverse orientation. COS refers to non-transfected cells. At dilutions higher than 1:20, no survival above control was seen with either COS- or COS conditioned medium. In all experiments without NGF, a monoclonal anti-NGF antibody (Korsching et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 80:3513-3516) was used at 1μg/ml.

As shown in Table V, only the medium obtained from cultures of COS cells bearing pCMV1-pBDNF plasmid in the sense orientation was associated with chick sensory neuron survival above control values. Therefore, the recombinant BDNF is biologically active. Further, addition of BDNF isolated from pig brain did not increase the survival of sensory neurons significantly above survival levels resulting from recombinant BDNF alone, indicating that the recombinant BDNF is capable of saturating BDNF receptors. Recombinant BDNF was also observed to have an additive or synergistic effect toward promoting chick sensory neuron survival when used together with NGF.

TABLE V

SURVIVAL OF CULTURED CHICK SENSORY NEURONS

COS medium (final dilution)	1:20	1:50	1:200
COS+		2,510±263	2,833±171
COS-	211±16		
COS	250±87		
BDNF + COS+		2,516±209	
NGF + COS+		5,770±72	

BDNF alone		2,718±424	

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11.   EXAMPLE: GENERATION OF ANTIBODIES TO BDNF

      11.1.   MATERIALS AND METHODS

A polyclonal antiserum specific for BDNF, designated sera 4, was generated in a NZ white rabbit by immunization with a synthetic peptide.

    11.1.1.   PEPTIDE SYNTHESIS AND COUPLING TO CARRIER

A peptide consisting of 34 amino acid residues, designated B5, was synthesized by conventional methods. It has the following amino acid sequence, corresponding to 33 residues of mature BDNF (residues 153 to 185 of the complete porcine preproBDNF sequence shown in Figure 1) with an additional cysteine residue at the amino-terminus (shown in italics) to permit coupling to a carrier protein using m-maleimidobenzoic acid-N-hydroxysuccinimide (MBS), if desired:

Cys-Val-Thr-Ala-Ala-Asp-Lys-Lys-Thr-Ala-Val-Asp-Met-Ser-
Gly-Gly-Thr-Val-Thr-Val-Leu-Glu-Lys-Val-Pro-Val-Ser-Lys-
Gly-Gln-Leu-Lys-Gln-Tyr

The B5 peptide was coupled to bovine serum albumin (BSA) using bis-diazo benzidine (BDB). Fresh BDB was prepared by dissolving 46 mg benzidine-HCl (p-diaminodiphenyl-HCl, obtained from Sigma) in 9.0 ml of 0.2 N HCl. 35 mg $NaNO_2$ was dissolved in 1.0 ml H_2O and added to the benzidine solution, and stirred for 1 hour at 4°C. 21 mg of BSA was dissolved in 3.0 ml of 0.16 M borate, 0.13 M NaCl, pH 9.0. Approximately 15 mg of B5 peptide was dissolved in 1.5 ml borate-NaCl buffer, pH 0.0. The peptide solution was added to the BSA solution, and placed in ice. 1.0 ml of BDB was added to the BSA-peptide solution, and the reaction mixture was incubated with stirring for 2 hours at 4°C; the pH was monitored and maintained in the range of 9.0 by the addition of small amounts of 0.5 M NAOH, as required. The reaction was terminated by addition of 0.2 ml of 1% phenol-buffered solution. Excess reagents were removed by dialysis against phosphate buffered saline (PBS).

11.1.2. IMMUNIZATION

A total of six rabbits were immunized according to the following protocol:

Rabbits 1 and 4--peptide B5 coupled at its C-terminus to BSA, using BDB;

Rabbits 2 and 3--peptide B5 coupled at its N-terminus to BSA, using MBS;

Rabbits 5 and 6--peptide B5 mixed with powdered nitrocellulose.

In all cases the first immunization used 1 mg of immunogen (100 μg B5/500 μg nitrocellulose for rabbits 5 and 6) in 0.5 ml PBS plus 0.5 ml ml complete Freund's adjuvant. This mixture was injected subcutaneously into

multiple sites on the back. The second immunization was
carried out three weeks later, and was identical to the
first except that incomplete Freund's adjuvant was used in
place of complete Freund's adjuvant. Subsequent boosts
occurred at intervals of 4-6 weeks. Rabbits were bled 1

5   week after immunization, and the antisera routinely checked
for binding to the pure B5 peptide by enzyme-linked
immunosorbent assay (ELISA).

11.1.3.   DETECTION OF ANTIBODY BINDING TO BDNF

10   100 μg of antigen (B5 peptide) in H_2O was added
to wells on a microtiter plate and allowed to dry
overnight, then washed briefly with H_2O and blocked with
100 μg 1% gelatin for 30 minutes at room temperature.
Wells were washed three times with distilled water, and

15   then 100 μg of antisera was added and allowed to incubate
at 4°C overnight. Wells were then washed three times in
PBS/0.05% triton X-100, after which 100 μg peroxidase
labeled anti-rabbit immunoassay (1/1000 dilution) was added
to wells and incubated at room temperature for three hours.

20   Wells were washed twice, and 100 μg ABTS solution (10 mg
ABTS (Sigma) dissolved in 10 ml 0.1 M NaCitrate pH 4.0 plus
10 μg H_2O_2) was added and incubated for about 5 minutes,
until color developed. The reaction was stopped by the
addition of 10 μg 1% NaN_3. Samples were diluted 1:5 with

25   H_2O and optical density was measured at 415 nm.

11.2.   RESULTS AND DISCUSSION

The antiserum from rabbit 4 (sera 4) showed the
highest titre, (Figure 7a) and was utilized in subsequent

30   experiments. Antibodies from sera 4 were partially
purified by precipitation with ammonium sulphate; to a
portion of antiserum an equal volume of saturated ammonium
sulfate was added slowly, with stirring, and the solution
was stirred for a further 15 minutes, then centrifuged at

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2,000 x g. The pellet was washed twice in 50% saturated
ammonium sulphate, then redissolved in PBS in a volume
corresponding to the original volume of serum. Ammonium
sulphate was removed by dialysis against several changes of
PBS. The dialyzed solution was aliquoted in 1.0 ml volumes
and subjected to lyophilization using a speed-vac. A
sample of sera 4 antibody was resuspended in 0.5 ml H_2O and
tested for reactivity with peptide B5 by ELISA, and was
found to react to a dilution of 1:4000.

Polyclonal antibodies to a synthetic peptide
(B5) corresponding to a 33 amino acid fragment of porcine
BDNF were generated by immunizing rabbits as described
above. Sera 4, which showed the highest titre against the
synthetic peptide, showed reactivity with purified BDNF
from porcine brain by ELISA (Figure 7b). Weak reactivity
was also detected by immunoblotting (DATA NOT SHOWN).
However, the antiserum was unable to block the activity of
BDNF in a biological assay on chick embryo dorsal root
ganglion sensory neurons.

12.   **EXAMPLE: NOVEL BIOLOGICAL EFFECTS OF BDNF**

The following observations indicate that BDNF is
capable of (i) sustaining the survival and inducing the
fully differentiated state of CNS dopaminergic neurons;
(ii) sustaining the survival of CNS cholinergic neurons;
and (iii) suppressing the proliferation of astroglial
cells. These biological effects of BDNF have not been
described previously. As dopaminergic neurons, cholinergic
neurons, and astroglial cells may each be associated with
neurological diseases or disorders, BDNF may prove to be
useful in the treatment of neuropathologies involving these
cell populations.

12.1.   MATERIALS AND METHODS

12.1.1.   METHODS FOR CULTURING DOPAMINERGIC SUBSTANTIA NIGRA NEURONS

Ventral mesencephalon was dissected from brains of rat embryos varying in age from embryonic day 13 to

5   embryonic day 15. Typically, two litters were used in each experiment. The dissection solution had the following composition: NaCl, 136.8 mM, KCl, 2.7 mM, $Na_2HPO_4 \cdot 7H_2O$, 8.0 mM, KH_2PO_4, 1.5 mM, glucose, 6 mg/ml, and BSA, 0.1 mg/ml, pH 7.4. This solution was prepared and subsequently filter

10   sterilized through a 0.2 μM pore filter. The dissection was performed under non-sterile conditions. Once the tissue was dissected from all the brains, the rest of the procedure was carried out under sterile conditions. The tissue fragments were placed in a 35 mm culture dish and

15   minced using a fine scissors. Two ml of F-12 nutrient media containing 0.125% trypsin was then added to the tissue, and an incubated at 37°C. At the end of this incubation period, DNAseI was added to the slurry such that the final concentration was 80 ng/ml. Another identical

20   incubation was carried out, and the tissue slurry was subsequently added to 8.0 ml of growth medium consisting of Minimal Essential Medium (MEM) supplemented with 2 mM glutamine, 6 mg/ml glucose, 5 units/ml penicillin, 5 mg/ml streptomycin, and 7.5% fetal calf serum (FCS). The sample

25   was centrifuged in a tabletop centrifuge at room temperature at 500 rpm for a period of 5 minutes. The medium was aspirated, and 2 ml growth medium was added to the cell pellet. A fire polished pipette with an opening of 1 mm was used to triturate the cells eight times. The

30   remaining tissue fragments were allowed to settle by gravity, and a small aliquot of the supernatant was taken to assess cell number by counting in a hemocytometer. After cell density was determined, the cells were plated into tissue culture plates at a density of 50,000/cm^2.

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The culture plates were prepared on the day prior to dissection. Tissue plates (24 well, 2 cm^2/well) were precoated with polyornithine (molecular weight 30,000-70,000 g/mol), 0.5 mg/ml, at room temperature for 3 hours. The plates were extensively washed with water, and subsequently treated with mouse laminin, 5 μg/ml, at room temperature for 3 hours. The plates were then washed with water as above, and incubated overnight at 37°C in a humidified atmosphere consisting of 5% CO_2, 95% air, in the presence of growth medium. The medium in the plates was removed the following day and replaced with fresh growth medium.

Once the cells were plated onto the culture plates, the cells were placed in an incubator set at 37°C and 5% CO_2/95% air for a period of 24 hours. The culture medium was changed to a serum-free formulation (SFM) having the following composition: a 1:1 (vol:vol) mixture of Basal Eagle Medium (BEM) and nutrient mixture F-12 with glucose (33 mM), glutamine (2 mM), $NaHCO_3$ (15 mM), HEPES (10 mM), supplemented with insulin (25 μg/ml), transferrin (100 μg/ml), putrescine (60 μM), progesterone (20 nM), sodium selenite (30 nM), penicillin (5 U/ml), streptomycin (5 mg/ml), and T_3 (30 nM). In some experiments, purified BDNF was added to the cultures after the media change to SFM on culture day 2.

The solutions used for culturing dopaminergic neurons were prepared using water taken from a Milli-Q reagent water system. The tissue culture media formulations were obtained through Gibco Laboratories (Santa Clara, California), as was the fetal calf serum (lot number 43N1086) and the mouse laminin. All other media components were purchased from Sigma Chemical (St. Louis, MO), and were cell culture tested grade. The polyornithine and DNAseI were also obtained from Sigma. Trypsin was obtained from Worthington (Freehold, NJ), lot number 3667.

Commercial chemicals were of analytical grade, purchased from Baker Chemical (Phillipsburg, NJ). The BDNF used in these experiments was purified from pig brain by Dr. Y.-A. Barde, according to the procedure of Barde et al., 1982 (supra).

12.1.2. METHODS FOR IMMUNOCYTOCHEMICAL STAINING OF VENTRAL MESENCEPHALON CULTURES

Fixative solutions were prepared fresh for each experiment. For the staining of tyrosine hydroxylase (TH), the fixative was 4.0% paraformaldehyde in Sorenson's phosphate buffer. The Sorenson buffer was prepared by adding a 0.2 M solution of KH_2PO_4 to a stock of 0.2 M Na_2HPO_4 until the pH reached 7.3. The paraformaldehyde was subsequently added to the solution and briefly heated, to allow it to be dissolved, and cooled to room temperature before use.

To begin the procedure, culture medium was removed from the culture dishes by gentle suction, and the proper fixative solution was gently added to the dish. A room temperature incubation of 20 minutes was carried out. Three washes in Sorenson's phosphate buffer, for 5 minutes each, with gentle rotation, followed. The cells were then incubated in a quench solution for 30 minutes at room temperature with gentle rotation. The quench solution for the cultures to be stained for TH consisted of Sorenson's phosphate buffer containing 2% normal horse serum. Next, the cultures were incubated in permeabilization buffer at room temperature for 30 minutes with gentle rotation. The solution consisted of Sorenson's buffer containing 0.2% saponin, and 1.5% of normal horse serum for the cultures to be stained for TH. Following the permeabilization step, the cultures were incubated in the presence of primary antibody overnight at 4°C. The antibody against rat TH was a mouse monoclonal antibody of isotype IgG2a. It was used

at 40 µg/ml in a solution of 10 mM $NaPO_4$, 50 mM NaCl, 0.2%
saponin pH 7.5. Following the primary antibody incubation,
the cultures were washed 5 times for 15 minutes each in the
appropriate permeabilization buffer. Next, the cultures
were incubated with secondary antibody conjugated to
5 biotin, that is biotinylated horse anti-mouse IgG. This
incubation was carried out at room temperature for two
hours with gentle rotation. Washes identical to those
described above followed, and the cultures were then
incubated in the presence of a preformed avidin-
10 biotinylated horseradishperoxidase complex (ABC reagent,
Vector Laboratories, Burlingame, CA) prepared according to
manufacturer's protocol. After a 30 min. incubation at
room temperature with gentle rotation, the cultures were
washed as described above. The cultures were subsequently
15 incubated with 55 mM Tris-Cl pH 7.3 containing 0.5 mg/ml
diaminobenzidine and 0.01% hydrogen peroxide. The
development of reaction product was allowed to proceed for
2-5 min. after which the solution was removed and the
cultures were washed several times with ice cold PBS. The
20 number of positive cells/cm^2 was then ascertained.

The paraformaldehyde and the glutaraldehyde were
obtained from Fluka Chemical. Vectastain kits containing
normal serum (used as a blocking agent), biotinylated,
affinity-purified anti-immunoglobulin, avidin DH, and
25 biotinylated HRP-H were purchased from Vector Laboratories.
The diaminobenzidine was obtained from BRL (Gaithersberg,
MD).

30 12.1.3.   METHODS USED IN MEASURING ^{3}H-DOPAMINE UPTAKE
          IN VENTRAL MESENCEPHALON CULTURES

[3]H-dopamine (^{3}H-DA) uptake was performed as
described by Dal Toso et al. (1988, J. Neurosci. 8:733-745)
with minor modifications. The uptake buffer had the
following composition: NaCl, 136.8 mM, KCl, 2.7 mM,
35

$Na_2HPO_4 \cdot 7H_2O$, 8.0 mM, KH_2PO_4, 1.5 mM, glucose, 5.0 mM, $caCl_2$2, 1.0 mM, $MgSO_4$, 1.0 mM, ascorbic acid, 0.1 mM, pargyline, 0.1 mM, pH 7.4 When necessary, 5.0 μM benztropine mesylate (BZT) was added to the uptake buffer.

   The cells were washed once with prewarmed (37°C) uptake buffer, and replaced with 0.4 ml uptake buffer/2 cm^2 well. The cultures were then preincubated for 5 min. at 37°C. At the end of this preincubation 0.1 ml ^{3}H-DA, in uptake buffer (250 nM, 40 Ci/mMol), was added such that the final concentration of ^{3}H-DA in the buffer was 50 nM. The cultures were incubated at 37°C for 15 min., followed by four washes at 4°C, 0.5 ml each, with uptake buffer. Two additional washes with ice cold PBS (10 mM $NaPO_4$, 150 mM NaCl, pH 7.6) were carried out as well. After the last wash was completed, 0.2 ml/2 cm^2 well 0.5 N NaOH was added to the cells, and allowed to stand at room temperature for two hours. The NaOH extract was subsequently collected, and counted in a scintillation counter (Packard, LS 500TD) with 10 ml "ultimagold" scintillation fluid. Specific uptake was defined as that uptake which was abolished in the presence of 5 μM BZT. Typically, this represented 70-90% of the total uptake observed.

   ^{3}H-DA was obtained from NEN (Boston, MA). Ascorbate, pargyline, BZT, and glucose were obtained from Sigma (St. Louis, MO). The ultimagold scintillation fluid was purchased from Packard (Sterling, VA).

12.1.4. METHODS FOR PRODUCING CULTURES OF BASAL FOREBRAIN CHOLINERGIC NEURONS

   Primary cultures of basal forebrain cholinergic cells were produced from embryonic day 17 rats. Specifically, the cholinergic neurons used in this study were derived from the medial septal nucleus and the nucleus of the diagonal band of Broca. This neuronal population projects primarily to the hippocampus. The dissociated

mixed cultures (neurons and glia) were produced by the following procedure. The septal region was dissected free from the surrounding tissue, and removed from the fetal brain. The tissue pieces were then pooled, minced with scissors, and treated for 20 minutes at 37°C with 12.5%

5 trypsin. The trypsin was inactivated by dilution in the plating medium (Dulbecco's modified Eagle medium (DMEM) containing 1% penicillin and streptomycin, 5% horse serum, and 1% N3, a hormone supplement). A single cell suspension was produced by trituration of the digested tissue

10 fragments with a fire polished pasteur pipette. The dissociated cells were counted on a hemocytometer and plated at the appropriate density in the plating medium. Test ligands were added to the cultures five to six hours after plating and the cells were then grown in vitro for

15 ten days with medium changes being made every three days.

        12.1.5. CHOLINE ACETYL TRANSFERASE ASSAYS
                Following the treatment period the cells were either used for choline acetyltransferase (CAT) enzyme

20 assays or processed for immunohistochemical staining for CAT by the following protocol. The monoclonal antibody to CAT was purchased from Boehringer Mannheim Biochemical Co. and has been characterized elsewhere. At the end of the experimental period the cells were rinsed twice with DMEM.

25 The cultures were fixed by a two step procedure; 50 μl of 4% paraformaldehyde was added to 50 μl of DMEM for a 10 minute incubation period. This solution was removed and replaced with 100 μl of 4% paraformaldehyde and the incubation was continued for 30 minutes at room

30 temperature. Following the fixation, the cells were rinsed three times with phosphate buffered saline (PBS) and permeabilized by a 30 minute incubation with saponin (0.5 mg/ml). The detergent was removed with three washes of

35 PBS, and a blocking solution of 5% normal rabbit serum was

added for 30 minutes. The primary antibody was added at a
dilution of 1:3 in 1% normal rabbit serum, subsequent to
the removal of the blocking solution, and the cultures were
incubated overnight at 4°C. The solution containing the
primary antibody was removed with a PBS wash. The bound

5   immunoglobulin was detected by the Vectastain "ABC" method.
Diaminobenzidene tetrahydrochloride (DAB) was used as the
substrate for the peroxidase reaction which was usually
carried out for one to five minutes. The reaction was
stopped by rinsing the cultures two times with 0.1 M tris-

10   HCl pH 7.2. The cultures were stored in 50 mM tris, pH 7.6
containing 0.15 M NaCl, 42% glycerol, and 0.15% Zephiran
(Pierce Chemical Co., Rockville, IL) at 4°C.

     12.1.6.   METHOD OF GENERATING PURIFIED
15   ASTROGLIAL CELL CULTURES
     Purified glial cultures were prepared
essentially by the method of McCarthy and DeVellis
(McCarthy, K. D., and DeVellis, J., 1980, J. Cell Biol.
85:890-902) from postnatal day 1-2 rat hippocampus.

20   Hippocampi were removed from five pups and minced with
scissors. The tissue pieces were then digested in 2 mls of
0.125% trypsin for 20 minutes at 37°C. The protease was
inactivated by dilution, with plating medium (10% fetal
bovine serum Gibco, DMEM, 0.5% penicillin (5000 mcg/ml) and

25   0.5% glutamine). A single cell suspension was produced by
passing the digested tissue fragments through a constricted
pasteur pipette. The cells were pelleted by
centrifugation at 900 rpm for five minutes, resuspended in
plating medium, and then counted on a hemocytometer. The

30   single cell suspension was divided into three, 75 cm^2
tissue culture flask and the cells were grown to
approximately 80% of confluency. The cells were then
subcultured by a trypsinization protocol similar to that

35

just described. The glial were counted and plated at a
density of 10,000 cells/0.9cm^2.

12.2. RESULTS

12.2.1. THE EFFECT OF BDNF ON TYROSINE HYDROXYLASE PRESENT IN VENTRAL MESENCEPHALON CULTURES

Immunocytochemical staining, as described in
section 12.1.2, supra, was used to measure the effect of
BDNF on the number of tyrosine hydroxylane (TH) positive
cells (Figure 8). A maximal increase of more than 200
percent of control was observed by day 8 in BDNF stimulated
cultures of ventral mesencephalon cells. A very. slight
increase was observed as early as culture day 3 in BDNF-
stimulated cultures.

12.2.2. THE EFFECT OF BDNF ON DOPAMINE UPTAKE UPTAKE BY VENTRAL MESENCEPHALON CULTURES

^{3}H-dopamine (^{3}H-DA) uptake was measured
according to the method of Dal Taso et al. (1988, J.
Neurosci. 8:733-745) with minor modifications, as described
in section 12.1.3, supra. A slight increase in dopamine
uptake was observed in BDNF stimulated ventral
mesencephalon cultures by day 8 in culture (Figure 9).

12.2.3. THE EFFECT OF BDNF ON CHOLINE ACETYLTRANSFERASE EXPRESSION BY FOREBRAIN CHOLINERGIC NEURONS

Figure 10(a) depicts the effect of BDNF on the
number of CAT positive cells following a growth period of
12 days in vitro. A 5.9 fold increase in CAT cell number
was observed with the addition of 100 ng/ml of BDNF, while
the EC50 was calculated to be 10 ng/ml. As a positive
control, cultures at 260,000 (Black bar) or 150,000
(hatched bar) cells per well were treated in an identical
manner with NGF (Figure 10(b)). The 260,000 cell per well
density corresponds to that used for the BDNF study. This
increase in the number of CAT immunopositive cells is

similar to that which has been reported previously. The
potential for BDNF to act on cholinergic neurons was also
examined by measuring CAT enzyme activity (F. Fonnum; J.
Neurochemistry, 1975, 24:407-409). Figure 11 depicts the
changes in CAT produced with a BDNF treatment. In this

5 case, a 1.8 fold increase was achieved with 100 ng/ml of
BDNF and the EC50 was calculated to be 61 ng/ml.

12.2.4.   EFFECTS OF BDNF OR EGF ON ASTROGLIAL CELL CULTURES

10   Type II astrocytes have been demonstrated to
have high affinity receptors for a variety of
neurotransmitters and neuropeptides. Thus astrocytes are
capable of responding to neuronally derived signals. For
this reason and because the type II astrocytes represent a

15 cellular component in the primary cultures, a possible
direct effect of BDNF on glial cells was tested. The cells
were kept in vitro for four days prior to the addition of
growth factors to allow them to reach approximately 60% of
confluency and were then treated for 42 hours with EGF or

20 BDNF. For the last 18 hours of the incubation
$[^3H]$methylthymidine was present in the medium. The effect
of EGF is shown in Figure 12(a). As has been previously
reported, EGF was found to be mitogenic for the astrocytes.
The maximal response was observed with 10 ng/ml of EGF

25 which produced at 5.2-fold increase in $[^3H]$methylthymidine
incorporation. Figure 12(b) depicts the effect of BDNF on
$[^3H]$methylthymidine incorporation. The response to BDNF
appears to be biphasic: very low doses (0.1 ng/ml)
produced a slight increase in thymidine incorporation;

30 while doses greater than 1 ng/ml BDNF inhibited the
incorporation of $[^2H]$methylthymidine. BDNF at a dosage of
5 ng/ml produced an inhibition of 24% suggesting a decrease
in the rate of glial cell proliferation over the treatment
period.

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12.3. DISCUSSION

These in vitro experiments clearly show that BDNF sustains the survival or induces the fully differentiated state of dopaminergic neurons of the developing rat substantia nigra, as shown by staining for tyrosine hydroxylase and dopamine uptake in cultures of these neurons derived from embryonic rat mesencephalon. As these are the neurons which degenerate in Parkinson's disease, it is highly probable that BDNF has therapeutic potential in Parkinson' disease either by reducing loss of neurons or by increasing the levels of tyrosine hydroxylase (the rate limiting enzyme in dopamine synthesis) or possibly both.

Furthermore, like nerve growth (NGF), BDNF appears to have an effect on the survival of cholinergic neurons of the rat basal forebrain as shown by increased choline acetyltransferase (CAT) staining, increased CAT activity and increased acetylcholinesterase staining in cultures of rat embryo medial septal nucleus and nucleus of the diagonal band of Broca. Accordingly, BDNF alone or in conjunction with NGF may be useful in the treatment of diseases or disorders that affect the cholinergic neurons of the basal forebrain, including, for example, Alzheimer's disease.

13. EXAMPLE: IDENTIFICATION OF A NOVEL GENE IN THE BDNF/NGF GENE FAMILY

The approach of identifying novel members of the NGF/BDNF gene family by utilizing PCR with degenerate oligonucleotides, based on the segments of amino acid sequence conservation between NGF and BDNF (Boxes 1-4, see section 5.8), was first tested by determining whether pairs of such primers could be utilized to amplify both NGF and BDNF gene sequences from genomic DNA of several species. This method was then used to identify a novel gene which

-96-

shares homology with NGF and BDNF in all four boxes of
homology noted.

13.1.   MATERIALS AND METHODS
13.1.1.   POLYMERASE CHAIN REACTION
5   PCR was performed essentially as described in
Section 6, supra.

13.2.   RESULTS
13.2.1.   AMPLIFICATION OF BOTH NGF AND BDNF
SEQUENCES FROM GENOMIC DNA
10   Degenerate synthetic oligonucleotide primers
were synthesized corresponding to portions of Box 1 and Box
2 of amino acid sequence conservation between NGF and BDNF
(see section 5.8 above), and utilized in a PCR reaction
with rat genomic DNA as template. The exact primer
15 sequences were as follows (positions of degeneracy, with
mixture of two or more bases included in oligonucleotide
synthesis step, shown in parentheses; underlining italics
indicate tails with multiple restriction endonuclease
cleavage sites provided to facilitate ligation to vector in
20 a subsequent cloning step; A=adenine, G=guanine,
C=cytosine, T=thymine, N=mixture of A,G,C,T):
          Box 1 (sense), primer 1B:
5'-GACTCGAGTCGACTCGGTGTG(C,T)GACAG(C,T)(A,G)T(C,T,A)AG-3'
          Box 2 (antisense), primer 2C:
25 5'-CCAAGCTTCTAGAATTCCA(C,T)TT(N)GT(C,T)TC(A,G)(A,T)A(A,G)AA
(A,G),TA(C,T)TG-3'
          300 ng of each degenerate primer mixture was
added to 500 ng of rat genomic DNA in 100 microliters of
the standard reaction mixture for PCR. 35 cycles were
30 carried out, each consisting of incubation for 1 minute at
94°C, 2 minutes at 43°C, and 2 minutes at 72°C. The
anticipated size of the product of PCR amplification of
either the BDNF or NGF gene using these primers would be
175 base pairs, including the two 17-mer "tails"
35

(underlined) included for convenience in subsequent cloning
steps. Electrophoresis of the reaction mixture on an 8%
polyacrylamide/5% glycerol gel yielded a major band of
amplified DNA of the anticipated size, 175 bp.

5   13.2.2.   DETECTION OF SEQUENCES COMPLEMENTARY TO THE BDNF/
                NGF PROBE IN GENOMIC DNAS OF VARIOUS SPECIES

               The 175 bp band was removed from the acrylamide
gel by electroelution, and amplified further in a second
PCR reaction of seven cycles, under identical reaction
10   conditions to the first except that the concentrations of
dGTP, dATP, and TTP were lowered to 50 μM each, and alpha-
^{32}P-dCTP tracer was utilized in place of unlabeled dCTP.
The radiolabeled DNA product was separated from reaction
components by chromatography on a sizing column. This
15   probe, designated "R1B/2C" (for rat DNA amplified from
primers 1B and 2C), was then utilized to detect
complementary sequences in genomic DNAs of various
vertebrate species (results with rat, mouse, and chicken
are shown in Figure 13), after digestion with EcoRI
20   restriction endonuclease and blotting to nitrocellulose by
the method of Southern hybridization on EcoRI-digested
genomic DNAs, as shown in Figure 13.

               The sizes of the EcoRI fragments of genomic DNA
containing NGF and BDNF sequences were determined in
25   controls on parallel blots using radiolabeled human NGF and
BDNF probes, prepared by PCR from cloned genes. The
positions of the NGF and BDNF genomic EcoRI fragments are
indicated in Figure 13 as N and B, respectively. The
results of a similar analysis were presented in Figure 3,
30   and equivalent results were obtained using a human BDNF
probe as with the porcine BDNF probe shown in Figure 3. As
noted above, NGF andBDNF probes each hybridized to single
EcoRI fragments in a variety of vertebrate genomic DNAs.
For example in rat DNA the BDNF probe detected a band of
35

approximately 8.8 kb, while the NGF probe detected a band
of approximately 10.4 kb.

       As seen in Figure 13, in every species tested
(data shown for chicken, mouse and rat) the R1B/2C probe
hybridized to a DNA band indistinguishable from that

5   identified by the NGF probe as well as to a band
indistinguishable from that identified by the BDNF probe
(in mouse, the NGF and BDNF genomic EcoRI fragments have
the same electrophoretic mobility, corresponding to
approximately 11.5-12.0 kb). This demonstrates that the

10   degenerate oligonucleotide primers 1B and 2C can be
utilized to amplify sequences from both the NGF and BDNF
genes. It was noteworthy that in some cases additional
bands were observed in the genomic Southern blot hybridized
with the R1B/2C probe. For example, in EcoRI-digested

15   mouse genomic DNA at least two additional bands (labeled X1
and X2, of approximately 19.0 kb and 1.5 kb, respectively),
corresponding to neither NGF nor BDNF, of were observed.
Similarly, at least two additional bands were observed in
hybridization to rat DNA (X1, X2, of approximately 7.3 and

20   1.2 kb, respectively), and at least one in chicken DNA (X,
approximately 2.6 kb). Additional bands, not labeled
explicitly in this figure, were also observed in some
cases. The presence of bands not attributable to NGF or
BDNF suggested the possible existence of additional

25   member(s) of the gene family. Similarly, bands clearly
distinct from those known to contain the BDNf and NGF gene
sequences were found utilizing other sets of primer pairs
and genomic DNA templates (data not shown).

30
         13.2.3.   IDENTIFICATION OF A NOVEL GENE
                   RELATED TO BDNF AND NGF
         A specific test of the hypothesis that novel
genes related to NGF and BDNF could be identified by PCR
using degenerate oligonucleotide primers was carried out

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using primers for Box 3 and Box 4 (see section 5.8 above),
and mouse genomic DNA as template. Degenerate primers were
synthesized of the sequences:

Box 3 (sense):
5'-GGGGATCCGCGGITG(T,C)(C,A)GIGGIAT(T,C,A,)GA-3'

Box 4 (anti-sense):
5'-TCGAATTCTAGATIC(T,G)IAT(AG)AAIC(T,G)LCCA-3'

(G=guanine, A=adenine, C-cytosine, T=thymine, I=inosine;
mixtures of more than one base at a single position in the
oligonucleotide shown in parentheses). Note that inosine
was utilized at some positions corresponding to the third
("wobble") base of a codon, to allow for the degeneracy of
the genetic code. It is also possible to utilize a mixture
of the four conventional DNA bases rather than inosine, and
essentially identical results have been obtained with such
primers.

With the degenerate Box 3/Box 4 primer pair, PCR
from the genomic NGF and BDNF sequences in mouse DNA would
be expected to amplify a segment of approximately 90 bp.
Utilizing the primers shown above, PCR was carried out for
four cycles with an annealing temperature of 45°C, followed
by 31 cycles with an annealing temperature of 49°C. The
products were analyzed by gel electrophoresis, and a major
band of the expected size was observed. In the mouse the
NGF gene contains a HindII restriction endonuclease
cleavage site in the region between Box 3 and Box 4, while
the BDNF gene contains a site of cleavage for teh enzyme
ApaI in this region. Therefore, digestion of the product
of PCR amplification with HindII and ApaI would be expected
to eliminate NGF and BDNF sequences from the major product
band. however, when the PCR product was digested to
apparent completion with these two restriction enzymes, a
digestion-resistant band was found to persist in the
amplified DNA. This suggests that, in addition to NGF and
BDNF genes, at least one novel gene had been amplified.

13.2.4. CHARACTERIZATION OF A NOVEL MEMBER OF THE BDNF/NGF GENE FAMILY

The digestion-resistant PCR product was eluted from a gel, and utilized as template in asymmetric PCR reactions in which either one of the original degenerate

5   primers was present in 100-fold molar excess over the other primer. This asymmetric amplification permitted the production of single-stranded DNA templates suitable for sequencing by the chain termination method. The sequence analysis of the novel gene (designated here as "M3/4" but

10 also referred to as Neurotrophin-3) was extended further by PCR amplification of sequences between an exact primer located between Boxes 3 and 4 and the polyA sequence at the 3' end of the gene's transcript, using the strategy for "rapid amplification of cDNA ends" (RACE) described by M.

15 A. Frohman, M. K. Dush, and G. R. Martin, Proc. Natl. Acad. Sci. USA, 85:8998-9002 (1988). The results of DNA sequencing revealed that a novel gene had been amplified, comprising an open reading frame capable of encoding a polypeptide distinct from both NGF and BDNF but closely

20 related in amino acid sequence to both (Figure 14).

Preliminary evidence that this new gene encodes a neurotrophic factor was obtained by determining its pattern of expression in rat tissues by northern blot hybridization. This analysis indicated that the novel gene

25 is expressed much more strongly in brain tissue than in any other tissue examined.

13.3. DISCUSSION

As shown above (Figure 13), the DNA probe

30 obtained by PCR amplification using primers for Boxes 1 and 2 with rat genomic DNA as template (R1B/2C) hybridized to novel bands in addition to those known to contain NGF and BDNF gene sequences in EcoRI-digested DNA of every species tested. A similar analysis was carried out using a

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radioactively labeled probe for the novel gene amplified
using Box 3 and 4 primers on mouse genomic DNA (M3/4). In
each case, the M3/4 probe hybridized to a single major band
of EcoRI-digested genomic DNA, that was distinct from the
bands known to contain BDNF and NGF sequences. It should e
5   noted that the EcoRI fragments of genomic mouse, rat, and
chicken DNA observed by hybridization with the M3/4 probe
are in every case coincident with one of the novel bands
obtained by hybridization with the R1B/2C probe. These are
the 19.0 kb EcoRI fragment in mouse DNA (X1 in Figure 14),
10   the 7.3 kb fragment in rat DNA (X1 in Figure 14), and the
2.6 kb band in chicken DNA (X in Figure 14). This suggests
that portions of the same gene were amplified from rat DNA
using the 1B/2C primer pair, and from mouse DNA using the
3/4 primer pair. Thus, at least one novel gene apparently
15   shares homology with NGF and BDNF genes in all four of the
boxes of homology noted above.

        The concept of the homology boxes between NGF,
BDNF, and additional members of the gene family (e.g.
"M3/4" also known as NT-3) has been expressed here
20   primarily in terms of primary amino acid sequence, and
methods for the identification of novel genes in the
family. However, it is important to note that there are
likely to be additional implications for the secondary and
tertiary structure of these neurotrophic factors, their
25   interactions with specific receptors, and rational design
of novel molecules with potential therapeutic value.

        For example, there are 6 Cys residues in NGF,
and all have been shown to be involved in disulfide bonds.
Numbering them from most N-terminal to most C-terminal as
30   Cys1 - Cys6, it is known that the disulfide bridges involve
Cys1-Cys4, Cys2-Cys5, Cys3-Cys6. The positions of all 6
Cys residues are conserved between NGF and BDNF, and the
positions of 3 Cys residues in the portion of "M3/4"
35   sequenced to date line up exactly with Cys4, Cys5, Cys6, of

NGF and BDNF. This suggests that the secondary structure
of all members of the gene family may be closely related,
and largely determined by the conserved Cys residues. It
should be noted that the homology boxes pointed out for
BDNF and NGF cover 5 of the 6 Cys residues (Cys1 in Box 1,
5   Cys2 in Box 2, Cys3 and Box 3, Cys5 and Cys6 in Box 4).
This supports the idea that these Cys residues and their
immediate neighbors play an important role in determining
the general structure of these neurotrophic factors. The
structural determinants for specific interaction with high
10 affinity receptors for each of the neurotrophic factors
presumably reside in unique portions of each molecule.

        Accordingly, novel chimeric genes may be
produced by recombination between family members (e.g. by
in vitro recombination, or by direct gene synthesis) at any
15 of the four homology boxes already described, or elsewhere
in the molecule. Such chimeric proteins are likely to have
similar secondary structure, because of conservation of Cys
residues, and other amino acid residues, but may have novel
biological properties. For example, a BDNF/NGF chimeric
20 protein may be bifunctional in terms of interaction with
both BDNF and NGF receptors. Chimeric proteins may also
differ from parent molecule(s) in terms of dimerization,
and other physico-chemical properties. Chimeric proteins
might also be able to function as antagonists of either
25 parent molecule.

        Active fragments of BDNF/NGF may be used to
design other family members based on knowledge of critical
"core" regions for appropriate folding, plus information
regarding which regions are required for type-specific
30 interaction with receptors.

        Comparison of new family members (e.g. "M3/4")
with already known family members may be used to reveal new
boxes of homology that can help guide the search for
additional members of the BDNF/NGF gene family. For
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example, "M3/4" comparison with BDNF reveals some useful homology boxes. One of particular interest involves the fourth conserved Cys residue, the only one not in the previously noted Boxes 1-4. There is actually a rather long segment of identity or conserved amino acid

5 substitution shared by BDNF and "M3/4", which includes this Cys residue, namely: His-Trp-Asn-Ser-Gln-Cys-(Arg or Lys)-Thr(Thr or Ser)-Gln-(Ser or Thr)-Tyr-Val-Arg-Ala-Leu= Thr. Within this region one could choose at least two homology boxes for the synthesis of useful degenerate

10 oligonucleotide primers (e.g. His-Trp-Asn-Ser-Gln-Cys requires only 96-fold degeneracy for an 18-mer primer, or 48-fold degeneracy for a 17-mer; Tyr-Val-Arg-Ala-Leu-Thr would also be a useful box).

15
14. EXAMPLE: INCREASED EXPRESSION OF BDNF IN NEUROBLASTOMA CELLS

14.1. MATERIALS AND METHODS

14.1.1. CELL LINES

CHP100, CHP126, CHP134, CHP234, LAN1, LAN5, NB9,

20 SY5Y, Y79, FO1, BU2, HO1, HL60, and COL320 are cell lines maintained in the laboratory of Dr. Fred Alt, who provided RNA for the Northern blot used in Figure 15. All cell lines were human tumor cell lines. CHP100 is a neuroepithelioma cell line; CHP126, CHP134, CHP234, LAN1,

25 LAN5, NB9, and SY5Y are neuroblastoma cell lines; Y79 is a retinoblastoma cell line; FO1, BU2, HO1 are melanoma cell lines, HL60 is a promyelocytic leukemia cell line, COL320 is a neuroendocrine colon carcinoma cell line.

30
14.1.2. PREPARATION OF RNA

RNA was prepared and Northern blots performed essentially as desribed in Section 8.1.1, supra, using a full-length human cDNA probe. 10 μg of total RNA was in each lane of the gel used to produce the Northern blot of

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Figure 15, except that the RNA in LAN1 was less heavily
loaded and for SY5Y one microgram of poly(A)$^+$ RNA was used.

14.2. RESULTS

Figure 15 depicts the results of Northern blot
analysis of BDNF probe hybridized to a panel of RNA samples
obtained from a variety of human cell lines. An abundance
of RNA which hybridized to BDNF probe was detected in
CHP234 and LAN5 cell lines, and lower amounts were found in
CHP126 and CHP134. All of the positive lines were derived
from human neuroblastoma tumors.

15. EXAMPLE: ACTIVITY-DEPENDENT REGULATION OF BDNF- AND NGF-mRNAs IN THE RAT HIPPOCAMPUS IS MEDIATED BY NON-NMDA-GLUTAMATE RECEPTORS

15.1. MATERIALS AND METHODS

15.1.1. TREATMENT OF RATS WITH KAINIC ACID

The rats usually received diazepam (Valium) 90
minutes after intraperitoneal injection of kainic acid
12mg/kg to suppress extensive seizure activity. As shown
in Fig. 19, Valium given after kainic acid did not
interfere with the further increase in BDNF- and NGF-mRNA
levels. Rats which did not receive Valium showed similar
increases of BDNF- and NGF-mRNA levels in hippocampus 3
hours after kainic acid, compared with animals given kainic
acid followed by Valium.

15.1.2. PREPARATION OF HIPPOCAMPAL CULTURES

Hippocampi were prepared from E17-day old rat
embryos, dissected and incubated for 20 minutes at 37°C in
phosphate buffered saline (PBS) without calcium or
magnesium ions, but containing 10mM glucose, 1mg/ml
albumin, 6μg/ml DNAase and 1mg/ml papain. After washing
with the solution without papain, the hippocampal cells
were carefully dissociated with a fire polished pasteur

pipette. Cells were collected by centrifugation at low
speed, resuspended in DMEM, supplemented with 10% fetal
calf serum and plated on plastic culture dishes (0.5 x 10^6
cells per 35 mm) which were precoated with poly-DL-
ornithine (0.5mg/ml) and laminin (5µg/ml). Three hours

5 after plating, the medium was changed to a serum-free one,
which contained the supplements as described by Brewer and
Cotman (Brain Res. 497:65, 1989), but lacking glutamate.
Neurons remained viable up to three weeks in culture and
were usually used for the experiments at day 7 after

10 plating.

            15.1.3.   AMPLIFICATION OF RNA
            Total cellular RNA was extracted as described by
Chomcynski and Sacci (Anal. Biochem., 1987, 162:156-159)

15 from 0.5 X 10^6 cells after the addition of a shortened NGF
cRNA recovery standard (30 fg). NGF mRNA and cRNA were
coamplified in a combined one tube reverse
transcription/polymerase chain reaction (RT/PCR) containing
1/5 of the extracted RNA, 1xRT/PCR buffer (10mM Tris-HCl pH

20 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.1mg/ml gelatin, 0.1% Triton
X-100), 0.25 mM dNTP, 0.1 µM each 5′ and 3′ primer, 5U
RNasin (Promega), 3.2 U AMV-reverse transcriptase (Life
Science) and 2U Taq Polymerase (Genofit) in a total volume
of 25 µl. The mixture was overlaid with mineral oil,

25 incubated at 41°C for 30 minutes, heated to 92°C for 60
seconds, primer annealing at 55°C for 60 seconds and primer
extension at 72°C for 60 seconds). The amplification
products (203 bp for NGF mRNA and 153 bp for recovery
standard) were separated on a 3% NuSieve/Agarose 3:1 gel

30 (FMC Bioproducts), alkali-blotted to a Hybond N plus
membrane (Amersham) and hybridized as described (Heumann,
R. and Thoenen, H., 1986, J. Biol. Chem. 261:9246; Lindhold
et al., 1988, J. Biol. Chem. 263:16348). For absolute

35 quantification, known amounts of in vitro transcribed NGF

mRNA and recovery standard were coamplified in parallel
reactions. Recently, a similar method was described by
Wang et al. (PNAS 86:9717-9721, 1989).

15.2. RESULTS AND DISCUSSION

5   BDNF and NGF are members of a gene family with
about 50% amino acid identity (Leibrock et al., 1989,
Nature 341:149). The molecules exhibit strictly conserved
domains. Contained within these domains are the 6 cysteine
residues most probably involved in the stabilization of the
10 three dimensional structure of these molecules, which is
necessary for their biological activity. However, there
are also variable domains in BDNF and NGF which determine
their different neuronal specificity (Lindsay et al., 1985,
Dev. Biol 112:319; Johnson et al., 1986, J. Neurosci.
15 6:3031; Hofer, M. and Barde, Y. 1988, Nature 331:261;
Rodriguez-Tebar et al., 1989, Dev. Biol. 136:296).
Moreover, the difference between these two neurotrophic
molecules is also indicated by their sites of synthesis.
NGF is expressed both in the periphery (Korsching, S. and
20 Thoenen, H., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:3513;
Ebendal et al., 1983, Exp. Cell Res. 148:311; Heumann et
al., 1984, EMBO J. 3:3183; Dhelton, D.L. and Reichardt,
L.F., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:7951) and in
the central nervous system (CNS) (Korsching et al., 1985,
25 EMBO J. 4:1389; Shelton, D.L. and Reichardt, L.F., 1986,
Proc. Natl. Acad. Sci. U.S.A. 83:2714; Whittemore et al.,
1986, Proc. Natl. Acad. Sci. U.S.A. 83:817; Large et al.,
1986, Science 234:352). The densities of innervation by
NGF-responsive neurons reflect the levels of NGF in the
30 corresponding target tissues (Korsching, S. and Thoenen,
H., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:3513; Ebendal et
al., 1983, Exp. Cell Res. 148:311; Heumann et al., 1984,
EMBO J. 3:3183; Dhelton, D.L. and Reichardt, L.F., 1984,
Proc. Natl. Acad. Sci. U.S.A. 81:7951; Korsching et al.,
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1985, EMBO J. 4:1389; Shelton, D.L. and Reichardt, L.F., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:2714; Whittemore et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:817; Large et al., 1986, Science 234:352). In contrast to NGF, BDNF is predominantly expressed in the CNS in neurons, and the

5   levels of BDNF-mRNA are considerably higher than those of NGF-mRNA, e.g. about 50-fold in the hippocampus. In the peripheral nervous system, NGF is synthesized by various non-neuronal cell types (Bandtlow et al., 1987, EMBO J. 6:891), whereas in the brain it is mainly localized in

10   neurons, as demonstrated by in-situ hybridization (Rennert, P.D. and Heinrich, G., 1986, Biochem. Biophys. Res. Commun. 138:813; Ayer-LeLievre et al., 1988, Science 240:1339; Whittemore et al., 1988, J. Neurosci. Res. 20:403). However, cultured type-1 astrocytes have also been shown to

15   produce substantial quantities of NGF (Lindsay, R.M., 1979, Nature 282:80; Furukawa et al., 1987, Biochem. Biophys. Res. Commun. 142:395). The relative contribution of neurons and astrocytes to the NGF synthesized in brain is not yet known.

20   In view of the predominant expression of both BDNF- and NGF-mRNAs in central nervous system neurons, we investigated whether the levels of these two mRNAs are influenced by neuronal activity and if so, which transmitter(s) could possibly be involved in the

25   regulation. In a first series of experiments, we prepared neuronal cultures from embryonic (E17) rat hippocampus.. As shown in Fig. 16a, depolarization of the hippocampal neurons with high (50mM) potassium resulted in an increase in BDNF-mRNA. Maximal levels were reached between 3 and 6

30   hours after increasing the potassium concentration. The potassium-mediated increase in BDNF-mRNA could be inhibited by omitting calcium ions from the medium and it was also reduced by inhibiting calcium influx by the calcium channel

35   blocker nifedipin (Fig. 16b).

Because the hippocampal cultures used consisted
of a mixed population of neurons exhibiting different
patterns of transmitter and receptor expression, we studied
the effect of various physiological and synthetic receptor
agonists on BDNF- and NGF-mRNA expression. The results
5   presented in Table VI show that of all the substances
tested, kainic acid, a glutamate receptor agonist (Monaghan
et al., 1989, Annu. Rev. Pharmacol. Toxicol. 29:365),
produced by far the largest increase in BDNF-mRNA in
hippocampal neurons. In contrast, other molecules, such as
10 carbachol (a muscarinic receptor agonist) and to a lesser
extent histamine and bradykinin, elevated slightly, but
significantly, BDNF-mRNA (Table VI). Because the levels of
NGF-mRNA in the hippocampal neurons were very low, we
established a quantitative polymerase chain reaction (PCR)
15 method suitable for determining changes in NGF-mRNA. Using
this method we found that, like BDNF-mRNA, NGF-mRNA levels
were also increased by potassium and kainic acid in
hippocampal neurons (Figure 17).

As shown in Figure 18, the maximal increase in
20 BDNF-mRNA in the hippocampal neurons was obtained with
about 25 μM kainic acid. A further increase in the kainic
acid concentration resulted in a decrease of the BDNF-mRNA
levels, which most probably reflects toxic effects of the
high concentrations of kainic acid on hippocampal neurons
25 (Figure 18). A glutamate receptor-mediated neurotoxicity
has previously been reported (Choi et al., 1987, J.
Neurosci. 7:357; Rothman, S.M. and Olney, J.W., 1987,
Trends Neurosci. 10:299; Choi, D.W., 1988, Neuron 1:623)
for various central neurons following the application of
30 analogues of the excitatory amino acid glutamate. However,
the concentrations of kainic acid necessary to enhance the
expression of BDNF- and NGF-mRNA in the hippocampal neurons
could clearly be separated from the concentrations
resulting in neurotoxicity.
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To investigate the effects of kainic acid in more detail, we studied whether the increase in BDNF-mRNA could be blocked by any of the known glutamate receptor antagonists (Monaghan et al., 1989, Annu. Rev. Pharmacol. Toxicol. 29:365). Kynurenic acid, a broad spectrum

5 glutamate receptor antagonist, as well as CNQX, a competitive inhibitor of non-NMDA receptors (Monaghan et al., 1989, Annu. Rev. Pharmacol. Toxicol. 29:365), completely blocked the kainic acid-mediated increase in BDNF-mRNA in hippocampal neurons (Table VII). On the other

10 hand, MK 801, which specifically blocks NMDA receptors, was ineffective in blocking the rise of BDNF-mRNA in these neurons. Moreover, NMDA itself did not change BDNF-mRNA levels (Table VI). This shows that kainic acid acts directly via its receptors and that the effect is not due

15 to the release of endogenous glutamate (which also acts on NMDA receptors). It can thus be concluded that kainic acid in vitro elevates BDNF-mRNA levels via non-NMDA glutamate receptors.

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TABLE VI

EFFECT OF VARIOUS RECEPTOR AGONISTS
ON BDNF-mRNA EXPRESSION IN CULTURED NEURONS

Addition	% of Control
none	100 ± 5
carbachol (50 μM)	220 ± 25
carbachol (50 μM) + atropine 10 (μM)	85 ± 15
nicotin (100 μM)	110 ± 7
histamine (50 μM)	150 ± 12
serotonin (100 μM)	95 ± 6
dopamine (100 μM)	115 ± 7
norepinephrine (25 μM)	75 ± 10
substance P (1 μM)	85 ± 9
somatostatin (1 μM)	110 ± 8
bradykinin (1 μM)	155 ± 15
kainic acid (25 μM)	1365 ± 70
NMDA (25 μM)	105 ± 10

TABLE VII

EFFECT OF ANTAGONISTS TO
THE DIFFERENT GLUTAMATE RECEPTORS ON
THE KAINIC ACID INDUCED EXPRESSION OF BDNF-mRNA

Addition	% of Control
none	100 ± 60
Kainic acid (25 μM)	1250 ± 60
Kynurenic acid (1 mM)	70 ± 6
Kynurenic acid (1 mM) + Kainic acid (25 μM)	84 ± 7
CNQX (10 μM)	109 ± 6
CNQX (10 μM) + Kainic acid (25 μM)	105 ± 7
MK-801 (5 μM)	96 ± 5
MK-801 (5 μM) + Kainic acid (25 μM)	1130 ± 80

To evaluate the physiological significance of our in vitro observations, we studied whether similar mechanisms were also operating in vivo. We treated adult Wistar rats of both sexes weighing 180-200g with 12mg/kg of kainic acid. After various time periods, we determined the changes in BDNF- and NGF-mRNAs in the hippocampus and cortex by Northern blot analysis. Fig. 19 shows that kainic acid elicited an increase in the levels of BDNF- and NGF-mRNAs in both brain regions. The increase in BDNF-mRNA was substantially larger than that of NGF-mRNA. Moreover, for as long as 24 hours after administration of kainic acid, the mRNA levels remained elevated. The time-course of BDNF- and NGF-mRNAs changes showed that the maximal increase in the hippocampus was reached approximately 3 hours after the administration of kainic acid (Fig. 19). It is interesting to note that the increase in BDNF- and NGF-mRNAs in the hippocampus was preceded by an increase in

c-fos-mRNA; the two phenomena may be causally related, as
was recently shown to be the case for sciatic nerve after
lesion (Hengerer et al., 1990, Proc. Natl. Acad. Sci.
U.S.A. 87:3899). Moreover, there is a delay in the
increase of both BDNF- and NGF-mRNAs in the cerebral cortex
5 compared with the increase in the hippocampus, which
indicates that the signals leading to an enhanced
expression of BDNF and NGF spread from the hippocampus to
other brain regions. This observation is in keeping with
previous reports (Morgan et al., 1987, Science 237:192)
10 showing that the increase in c-fos elicited by the
convulsant metrazole occurred first in the hippocampus and
then in the cortex.

        After previous studies had provided evidence
that NMDA receptors are involved in the regulation of
15 hippocampal mRNAs, such as NGFI-A (Cole et al., 1989,
Nature 340:474), we then studied whether or not the NMDA
receptor antagonists MK 801 and ketamine could block the
increase in BDNF- and NGF-mRNAs in vivo. However,
confirming our results in vitro, MK 801 did not inhibit the
20 kainic acid-mediated increase in hippocampal BDNF-mRNA,
although it effectively suppressed seizures resulting from
NMDA receptor activation (Fig. 20). Most probably this
NMDA receptor activation results from endogenous glutamate
released by kainic acid (Biziere, K. and Coyle, T.,
25 Neurosci., 1978, Lett. 8:303; McGeer et al., 1978, Brain.
Res. 139:381). Likewise, the increase in NGF-mRNA in
hippocampus after kainic acid treatment was neither
prevented by MK 801 nor by ketamine. In a previous study
(Gall, C.M. and Isackson, P.J., 1989, Science 245:758), it
30 was shown that electrolytical lesions causing limbic
seizures elevate NGF-mRNA in rat hippocampus. However, the
present results obtained with MK 801 and kainic acid showed
a clear dissociation between induction of seizures and
increases in BDNF and NGF expression. Diazepam (Valium)
35

treatment of rats prior to kainic acid injections
completely blocked the increase in BDNF- and NGF-mRNAs in
the rat hippocampus (Figure 20). The blocking effect of
diazepam on the increase of BDNF- and NGF-mRNA most
probably resulted from the suppression of neuronal activity
5   via the inhibitory GABAergic system. However, 90 minutes
after the administration of kainic acid (i.e.,
approximately 30 minutes after the start of convulsion),
the increase in BDNF- and NGF-mRNA could no longer be
blocked by diazepam, indicating that a relatively short
10 period of augmented activity may be sufficient to initiate
the cascade of events leading to an augmented expression of
the mRNAs of the two neurotrophic factors.

      The present investigation showed that non-NMDA
receptors may be involved in the regulation of BDNF- and
15 NGF-mRNA in hippocampus, thus distinguishing this
regulatory mechanism from that previously described by Cole
et al. (Cole et al., 1989, Nature $\underline{340}$:474) which concluded
that hippocampal mRNAs encoding early genes seemed to be
regulated via the NMDA-subtype of glutamate receptors. It
20 has been reported that some of the effects of kainic acid
on neurons can also be mediated by the quisqualate type of
glutamate receptors (Monaghan et al., 1989, Annu. Rev.
Pharmacol. Toxicol. $\underline{29}$:365).

      In conclusion, the present study showed that
25 neuronal activity regulates the levels of mRNAs encoding
the neurotrophic factors BDNF and NGF in the rat
hippocampus and cortex. Of all the substances tested,
kainic acid, acting via the non-NMDA glutamate receptors,
elevated BDNF- and NGF-mRNAs both in vivo and in
30 hippocampal neuronal cultures. In contrast, in the
periphery there is no evidence that NGF synthesis in non-
neuronal cells is regulated by conventional transmitter
substances or neuropeptides released by innervating (NGF
responsive) neurons (Barth et al., 1984, J. Cell Biol.
35

99:839; Hellweg et al., 1988, Exp. Cell Res. 179:18). BDNF
is predominantly expressed in the CNS and there is at least
a partial overlap between the central pathways using
glutamate as a neurotransmitter and regions shown to be
relatively rich in BDNF- and NGF-mRNA (Hofer et al., EMBO
5   J., in press; Monaghan et al., 1989, Annu. Rev. Pharmacol.
Toxicol. 29:365). In particular, there are striking
similarities between the distribution of NGF-mRNA as
demonstrated by in situ hybridization (Hofer et al., EMBO
J., in press) and kainic acid receptors visualized by
10 autoradiography in hippocampus, cerebral cortex, and
cerebellum (Monaghan et al., 1989, Annu. Rev. Pharmacol.
Toxicol. 29:365). The observations are compatible with the
interpretation that glutamate represents the physiological
transmitter regulating BDNF- and NGF-mRNAs in the CNS.
15

16. EXAMPLE: BRAIN-DERIVED NEUROTROPHIC
FACTOR INCREASES SURVIVAL AND
DIFFERENTIATED FUNCTIONS OF RAT SEPTAL
CHOLINERGIC NEURONS IN CULTURE

16.1. MATERIALS AND METHODS

20 16.1.1. PREPARATION OF DISSOCIATED CELLS
AND CELL CULTURE CONDITIONS

The septal region from rats (Sprague-Dawley)
after 17 days of gestation was dissected free from the
surrounding tissue. Tissue fragments were pooled, washed
25 three times with Ham's F-10, and then transferred to a 35mm
tissue culture dish and minced. A single cell suspension
was made by incubating the tissue with 0.25% trypsin for 20
minutes at 37°C. Following the inactivation of the trypsin
by a five minute incubation at room temperature in growth
30 medium (infra), containing 50ug/ml deoxyribonuclease type 1
(Sigma), the cells were dissociated by passing the fragments
repeatedly through the constricted tip of a Pasteur pipet.
The dissociated cells were then centrifuged at 500xg for 45
seconds. The supernatant was removed and recentrifuged.
35

The loose cell pellets were resuspended and combined in growth medium, and the cell yield was determined by use of a hemocytometer. Finally, the cells were plated into 6mm wells which had been coated with polyornithine (10μg/ml) and laminin (10μg/ml). The cell viability was checked, after 24

5   hours in culture, by the ability of the cells to exclude trypan blue.

The normal growth medium, 5HS/N3, for cultures composed of neurons and glia contained: 5% (v/v) horse serum (Gibco), 1% N3 additives (v/v) (Romijn et al., 1982,

10 Dev. Brain Res. 2:583-589), 0.5% (v/v) glutamine (200mM, Gibco), and 0.25% (v/v) penicillin-streptomycin (10,000 units/ml, 10,000 mcg/ml respectively, Gibco) in Dulbecco's modified Eagle's medium (DMEM). Neuronal-enriched cultures were prepared by replacing the growth medium, five to six

15 hours after plating, with DMEM containing: 1% N3 additives, 0.5% glutamine, and 0.25% penicillin and streptomycin. In both conditions, treatment with cytosine arabinoside (1μM for 24 hours) was used to limit glial cell proliferation.

20   16.1.2. ASSAY OF CHOLINE ACETYLTRANSFERASE ACTIVITY

The growth medium was removed from the cultures by rinsing the cells twice with 100μl of PBS. The cells were lysed via one freeze-thaw cycle and a 15 minute incubation at 37°C in 50mM KH_2PO_4 pH 6.7 containing 200mM

25 NaCl and 0.25% (v/v) Triton x-100. Two microliters of the cell lysate was removed and assayed for CAT activity according to the micro-Fonnum procedure (Fonnum, F, 1975, J. Neurochem. 24:407-409). The final substrate composition consisted of 0.2mM [^{14}C] Acetyl-CoA (NEN, 54.4, mCi/mmol),

30 300mM NaCl, 8mM choline bromide, 20mM ethylenediaminetetraacetic acid, and 0.1mM neostigmine in 50mM NaH_2PO_4 (pH 7.4) buffer. At these enzyme and substrate concentrations, the enzymatic reaction was linear for 90-120

35 minutes. The specificity of the induction for choline

acetyltransferase was tested by the addition of a specific
inhibitor of CAT activity, N-hydroxyethyl-4-(1-
napthylvinyl)pyridium (HNP), during the assay (White, H.L.
and Cavallito, C.J., 1970, J. Neurochem. 17:1579-1589).

5   16.1.3. ASSAY OF ACETYLCHOLINESTERASE (AChE) ACTIVITY
             The level of AChE present in the lysates was
measured by the methods of Potter (Potter, L.T., 1967, J.
Pharmacology and Experimental Therapeutics 156:500-506) and
Johnson and Russell (Johnson, C.D. and Russell, R.L., 1975,
10 Anal. Biochem. 64:229-238) with several modifications.
Lysates prepared in 50mM KH_2PO_4 buffer pH 6.7 containing
200mM NaCl and 0.25% (v/v) Triton x-100, were combined with
$[^3H]$ acetylcholine iodide (NEN, NET-113, 73.3 mCi/mmol),
ethopropazine (0.1mM) in 50mM KH_2PO_4 pH 7.0. Following a 15
15 minute incubation at 37°C, the reaction was terminated by
the addition of 100µl of a stop buffer solution containing:
1M chloroacetic acid, 0.5M NaOH, and 2.0M NaCl. Specific
AChE activity was determined in the presence of 1.0 (10^{-6})M
neostigmine.
20

       16.1.4. MEASUREMENT OF HIGH AFFINITY CHOLINE UPTAKE
             Choline uptake via a high affinity, Na+-dependent
mechanism was assayed essentially by the procedure of Vaca
and Pilar, 1979 (Vaca, K. and Pilar, G., 1979, J. Gen.
25 Physiol. 73:605-628). The cells were washed once with 100
µl Ham's F-10, followed by a 100 µl rinse with Tyrodes
solution. Following a 10 minute depolarization induced by
Tyrodes solution containing 25mM potassium, the cells were
incubated for 20 minutes at room temperature with $[^3H]$
30 choline (NEN, NET-109, 0.11µM, 86.7 Ci/mmol) in normal Na+
or Li+ containing Tyrodes solution. The uptake reaction was
stopped by rinsing the cells twice with PBS. The
accumulated choline was extracted by incubating the cultures
for at least 20 minutes with 100 µl of cold acetone
35

containing 1N formic acid. The soluble fraction was then removed and counted. The specific choline uptake is the difference between the level of total and the Na+-independent choline uptake.

5   16.1.5. HISTOCHEMICAL STAINING FOR ACETYCHOLINESTERASE
        Cholinergic cells were identified by histochemically staining for acetylcholinesterase by a modification of the staining method of Geneser-Jensen and Blackstadt, (1971, Z. Zellforsch. 114:460-481). Following

10   fixation of the cultures in 4% paraformaldehyde, the cells were incubated 5-6 days at 4°C in the presence of the AChE substrate solution composed of the following: 4mM acetylithiocholine iodine, 2mM copper sulfate, 10mM glycine and 10 μg/ml gelatine in 50mM acetate buffer pH 5.0.

15   Visualization of the reaction product was accomplished as previously described (Hartikka, J. and Hefti, F., 1988, J. Neurosci. 8:2967-2985).

        16.1.6. IMMUNOHISTOCHEMICAL STAINING FOR THE NGF-RECEPTOR
20   Cultures were washed twice with DMEM and then fixed with 4% paraformaldehyde. The cells were then treated for 3-4 hours with a sodium phosphate buffer pH 7.4 containing 5% bovine serum albumin, 0.02% triton x-100 and 5% sucrose (Hartikka, J. and Hefti, F., 1988, J. Neurosci.

25   8:2967-2985). The NGF-R was detected utilizing the monoclonal antibody 192-IgG (Taniuchi, M. and Johnson, E., 1985, J. Cell Biol. 101:1100-1106; Chandler et. al., 1984, J. Biol. Chem. 259:6882-6889) and at 1:1000 dilution in buffer containing 5% horse serum. The cultures were

30   incubated with the diluted antibody for 15-18 hours at 4°C. The bound mouse immunoglobulin was detected utilizing biotinylated horse antimouse IgG (1:200 Vector). The immunoreactive cells were identified by using 3',3'

35

diaminobenzidine as the substrate for the bound peroxidase enzyme.

16.1.7. PURIFICATION OF BDNF AND NGF

The purification of BDNF, from porcine brain, and
5 NGF, from male mouse submaxilliary gland, was conducted
according to previously published protocols (Barde et al.,
1982, EMBO J. 1:549-553; Lindsay et al., 1985, Dev. Biol.
112:319-328). The recombinant BDNF, used for a portion of
these studies, has been characterized previously
10 (Maisonpierre et al., 1990, Science. 247:1446-141).

16.2. RESULTS

Cultures of septal cells produced elaborate
neurite outgrowth on polyornithine-laminin coated wells,
15 with many of the cells displaying a characteristic neuronal
phase-bright soma by 24 hours (Figure 21A and B). Continued
neurite outgrowth resulted in a progressive increase in the
extent of cell-cell contact at days 2 and 4 in vitro
(compare Figure 21 C, D with E, F). There were very few
20 non-neuronal cells in the cultures even after 4 days, as
noted by the absence of flattened phase-dark cells. To
assess the effects of BDNF on septal cholinergic neuron
survival in culture, a histochemical stain for AChE and a
immunohistochemical stain for the NGF-receptor was used.
25 Typical morphologies of AChE positive neurons are shown in
Figure 21G, H. Several NGF-receptor positive neurons are
shown in Figure 21I.

BDNF elicited a 2.4-fold increase in the number
of AChE positive cells in low density cultures (defined as
30 cell densities between 66,700 - 133,300 cells/cm^2) of
embryonic rat septal cells as shown in Figure 22A. At the
same cell densities the effect of NGF was slightly less
(1.9-fold). The finding that BDNF as well as NGF can
produce an increase in the number of AChE positive neurons
35

in septal cultures prompted us to examine whether these two factors act upon similar or distinct neuronal populations. As shown in the third panel of Figure 22A, concurrent addition of saturating levels of BDNF and NGF lead to no greater increase in the number of AChE positive neurons than

5 that seen with either factor alone. In terms of neuronal survival, these results suggest that the cholinergic neurons in rat septal cultures which respond to BDNF or NGF must be closely overlapping populations.

The effect of BDNF upon the survival of AChE

10 positive neurons was dependent upon the density at which the cells were grown (Figure 22A). At high plating densities (200,000-266,000 cells/cm^2)neither BDNF alone nor in combination with NGF produced an increase in the number of AChE expressing neurons. This may be due to increased

15 endogenous levels of neurotrophic activity, or alternatively, survival promoting effects of the increased cell-cell contact occurring at the higher cell densities.

At low cell density, the number of AChE positive cells increased as a function of BDNF concentration, with a

20 maximal response observed at a dose of 10 ng/ml, which increased the number of AChE positive cells/well from control values of 169.2±12.9 to 388.0±26.2 (a 2.5 fold increase) in treated cultures. In comparison, a maximal response to NGF was observed at 50 ng/ml and produced an

25 increase in AChE positive cells/well from 121.0±7.6 to 231.7±12.9 (a 1.9 fold increase). The plateau phase in the response curve appeared to be stable for both BDNF and NGF, in that there was no reduction in the number of AChE positive neurons at doses as high as 100 ng/ml.

30 It is apparent that in low density cultures, both BDNF and NGF are capable of increasing the number of cells detected by AChE histochemistry. It is possible that this increase is due to a rescue of cholinergic cells dying in

35 culture in the absence of growth factor, to recruitment of

precursor cells, or to the induction of the cholinergic
marker, AChE. To attempt to differentiate among these
possibilities, delayed addition experiments were conducted
(Figure 23) in a series of cultures maintained for a total
of 12 days. One set of cultures, treated five to six hours

5 after plating, was maintained in the presence of BDNF or NGF
for the entire 12 days. A second set of cultures was
established without growth factors for 5 days and then
treated with BDNF or NGF for the last seven days (-5/+7).
When comparing these two sets of cultures, the response to

10 NGF or BDNF added after 5 days was much the same as was
observed when the cells were grown continuously in the
presence of the factors (compare solid and cross hatched
bars in Figure 23). The results were strikingly different,
however, in a third set of cultures in which BDNF or NGF was

15 only present for the final 5 days (-7/+5) cultures). Under
these conditions, neither NGF nor BDNF was capable of
enhancing the number of AChE positive cells. In other
experiments, it has been established that exposure of septal
neurons to either NGF or BDNF for as little as 3-4 days is

20 sufficient to produce a large increase in AChE enzyme
activity, and thus detection of cells by AChE staining.
Therefore, the lack of a detectable increase in the number
of AChE positive neurons in the "-7/+5" cultures was not due
to the possibility that the time of exposure to growth

25 factor was insufficient to allow for an induction of AChE
positive cells.

It has been shown _in vivo_ (Montero, C. and Hefti,
F., 1988, J. Neurosci. 8:2986-2999; Higgins et al., 1989,
Neuron. 3:247-256) as well as _in vitro_ that NGF is capable

30 of regulating the levels of its own receptor as measured
both at the mRNA and protein level. Since BDNF was found to
display similar effects to NGF in the induction of
cholinergic phenotype and cell survival in septal cultures,
the potential that BDNF may regulate the levels of NGF-

35

receptor, as quantitated by the numbers of IgG-192 immunopositive cells, was tested. At 10 ng/ml, BDNF produced a 3-fold increase in the number of NGF-receptor immunopositive cells, as shown in Figure 24. Interestingly, at higher concentrations (25-100 ng/ml) the effect of BDNF

5   was progressively less marked. Thus, the dose response in this instance was notably different from that observed for the effect of BDNF on other parameters in these cultures, e.g., number of AChE positive cells. As a positive control, the cells were treated with NGF at a concentration of 50

10   ng/ml, which also resulted in a 3-fold increase in the number of positive cells. Therefore, on the basis of IgG-192 staining, BDNF has an approximately equal effect to NGF on up-regulating the expression of the NGF-receptor.

        In addition to effects on cell survival, the
15   possibility that BDNF could promote cholinergic phenotypic traits was investigated. A linear increase in CAT activity was found in septal cultures grown in the presence of increasing levels of BDNF up to 50 ng/ml, where the response saturated at a level of 1.8 fold over control values, as

20   shown in Figure 25. Induction of CAT activity in parallel cultures treated with NGF reached a plateau at 25 ng/ml, representing a 3.4 fold increase over control values. The response to either BDNF or NGF showed no significant decline at three times the concentration sufficient to produce a

25   saturating response. The induction of CAT activity with either BDNF or NGF did not effect the level of [N-hydroxyethyl-4-(1-napthylvinyl) pyridium] HNP-independent transferase activity.

        In light of the finding that BDNF as well as NGF
30   elicited increased CAT activity in septal cultures, the response to concurrent treatment with both factors was studied (Table VIII). For these experiments, two concentrations of BDNF were used, 5 and 25 ng/ml, which

35   increased CAT activity by 1.4 and 2.6 fold, respectively.

When NGF (50 ng/ml), which alone increased enzyme activity
by 2.8 fold, was combined with BDNF, the level of CAT
activity reflected at least an additive effect (Table VIII).
At the concentrations of BDNF used, a purely additive effect
to that of NGF would have lead to increases of 4.2 and 5.4
5 fold, respectively, over control values. The observed
values were actually somewhat more than additive.

TABLE VIII

10
EFFECTS OF CO-ADDITION OF BDNF
AND NGF ON CAT ACTIVITY

Concentration (ng/ml)	CAT Activity (pmol/Hr/Well)	% Control
Control	561.3 ± 34.8	------
NGF(50)	1546.3 ± 89.7	280
BDNF(5)	768.0 ± 25.4	140
BDNF(25)	1470.1 ± 66.2	260
NGF(50) + BDNF(5)	2767.6 ± 177.2	490
NGF(50) + BDNF(25)	3742.0 ± 669.3	670

Septal cells were grown for a period of 12 days in 5HS/N3 in
the presence of the indicated concentrations of trophic
25 factors. CAT activity was determined as described. Values
represent the means $\pm$ S.E.M. of 4-5 determinations.

        The possibility that the biological response
30 observed with BDNF treatment was due to a BDNF-dependent
release of endogenous NGF was tested. To examine this
question, a monoclonal antibody to NGF (antibody 27/21,
Korsching and Thoenen, 1983) was used to block any NGF-
related response. As shown in Table IX, the effect of NGF

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on CAT activity was greatly reduced by anti-NGF, while the
BDNF-induced response was essentially unaffected.   One
should note, however, that the basal level of CAT activity
was reduced by about 20% in cultures treated with anti-NGF
alone, as compared to untreated controls.   Nevertheless, the
5 observed increase in CAT activity in septal cultures
produced by BDNF appears to be a direct effect and not
mediated by an increase in endogenous NGF.

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TABLE IX

EFFECT OF ANTI-NGF ON THE ABILITY OF
NGF AND BDNF TO INDUCE CAT ENZYME ACTIVITY

| | CAT Activity | |
Concentration (ng/ml)	(pmol/HR/well)	% Control
Control	517.53±3.2	___
BDNF(25)	1021.6±106.1	197
NGF(50)	1139.0±99.1	220
Control + Anti-NGF	418.7±27.0	81
BDNF + Anti-NGF	819.2±68.8	158
NGF + Anti-NGF	551.0±27.0	107

Following a 12 day growth period in 5HS/N3 in the presence
of the indicated concentrations of trophic factor the septal
cells (plated at a density of 2.3 (10^5) cell/cm^2) were
harvested. CAT activity was determined as described in the
Experimental Procedures section. Values represent the mean
± S.E.M. of 6 determinations.

The possibility that AChE activity is
coordinately regulated along with CAT activity by BDNF or
NGF was also tested (Figure 26). In contrast to CAT
activity, the dose response of AChE to BDNF or NGF appeared
quite similar in that the enzyme activity increased linearly
up to a concentration of 50 ng/ml. At this concentration,
NGF and BDNF increased AChE activity 274% and 234%
respectively, as compared to the non-treated control values.
The time course of BDNF or NGF induced increase
in CAT activity was investigated (Figure 27). CAT activity
in cultures treated with 50 ng/ml of BDNF increased to 170%
of control values by 3 days. This increase continued until
day 6, where it plateaued at 2.5-fold above control values

throughout the remainder of the period tested. In contrast, the CAT response to NGF (50 ng/ml) administration was more rapid, with the induced level reaching 2.5 fold over control by day 3. The increase in CAT activity continued to rise linearly, reaching a value of 3.2 fold over the controls

5   after 12 days.

Astrocytes, grown in culture for a period of time, have been shown to synthesize a variety of neurotrophic activities, in addition to NGF (Lindsay, R.M., 1979, Nature 282:80-82; Lindsay et al., 1982, Brain Res.

10   243:329-343; Alderson et al., 1989, Dev. Brain. Res. 48:229-241). Although we have ruled out the possibility that our present observations on BDNF are mediated through an increase in levels of NGF, it is conceivable that BDNF may act indirectly by modulating expression of other

15   neurotrophic factors, especially in glial cells. To assess the possibility that nonneuronal cells might influence the effect of BDNF on CAT activity in septal cultures, we compared the response of cholinergic neurons to BDNF in mixed glial-neuronal cultures and neuron-enriched cultures

20   (Figure 28). In neuron-enriched cultures, the response of CAT activity to BDNF displayed a bell-shaped curve; a maximal increase was achieved at a dose of 5 ng/ml of BDNF and at a concentration of 25 ng/ml there was a significant decline in enzymatic activity ($p > 0.001$, comparing 15 to 25

25   ng/ml of BDNF). Similar to the result shown in Figure 25, the CAT response to BDNF in the presence of a confluent glial layer was linear, in this case up to 25 ng/ml of BDNF, the highest dose tested. In the presence of nonneuronal cells higher levels of BDNF were required to produce an

30   equivalent increase in CAT activity to that found in similarly treated neuron-enriched cultures. Notwithstanding, the ability of BDNF to maximally stimulate CAT activity in the septal cholinergic neurons was

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essentially the same in the presence or absence of glial
cells.

On the basis of CAT and AChE activity, it is
apparent that BDNF can act similarly to NGF in enhancing
cholinergic phenotypic markers. To examine this further,
5 the effect of BDNF on the level of Na+-dependent, high-
affinity choline uptake was compared to that of NGF (Figure
29). Cells grown for 12 days in the presence of 50 ng/ml of
BDNF accumulated choline to a level 3.8 fold over that of
the non-treated controls. In parallel cultures, NGF (25
10 ng/ml) induced a 2.3 fold increase in choline accumulation.

16.3.   DISCUSSION

In comparison to its established effects on
peripheral neurons (Barde et al., 1982, EMBO J. 1:549-553;
15 Lindsay et al., 1985, Dev. Biol. 112:319-328; Davies et al.,
1986, J. Neurosci. 6:1897-1904; Hofer, M, and Barde, Y.,
1988, Nature 331:262-262), the neuronal specificity of BDNF
within the central nervous system is less well
characterized. To date, the only known responsive CNS
20 neurons are a small subpopulation of Thy-1 positive retinal
ganglion cells first identified in cultures of E17 rat
retina (Johnson et al., 1986, J. Neurosci. 6:3031-3038).
Further studies have also established effects of BDNF upon
adult retinal ganglion cells in explant cultures (Thanos et
25 al., 1989, Eur. J. Neuro. 1:19-26). In this study, we have
shown that BDNF increases the survival of cultured
cholinergic neurons from the embryonic rat septum, as shown
by AChE histochemical staining, and increases the expression
of various cholinergic phenotypic markers, i.e., enzymatic
30 activity of AChE and CAT and high affinity choline uptake.
In addition, BDNF was found to increase expression of the
NGF receptor in septal cultures.

In initial experiments, we found that BDNF
increased the number of AChE positive neurons in E17 septal
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cultures by approximately 2 fold. As with NGF ((Hartikka,
J. and Hefti, F., 1988, J. Neurosci. 8:2967-2985) this
effect was most apparent at low cell densities.
Interestingly, co-addition of NGF and BDNF were ineffective
in further increasing the number of AChE positive cells over
5   the level observed in the presence of either growth factor
alone. This observation strongly suggests that BDNF and NGF
act largely upon the same population of septal cholinergic
neurons.

As initially reported for NGF (Hefti et al.,
10 1985, Neuroscience 1:55-68), BDNF did not enhance
cholinergic neuron survival in cultures established at
relatively high cell densities. As a proportion of the
cells plated, cholinergic neuron survival was found to be
higher at high cell density than at low cell density, even
15 in the absence of any neurotrophic factor. There are
several possible explanations that may account for this
including increased cell-cell contact, beneficial effects of
increased numbers of nonneuronal cells or a more than
proportional increase in endogenous neurotrophic activity.
20 In terms of the latter possibility, we found no indication
of substantially increased levels of endogenous NGF since
addition of an excess of anti-NGF to untreated high density
cultures only reduced basal CAT activity by 20%. In such
cultures, exogenous NGF was able to produce as much as 3.4
25 fold increase in CAT activity.

As observed both at the level of protein and
mRNA, there are now many reports which indicate that NGF can
upregulate the expression of its own receptor both on
cultured neurons and in vivo. In this study, we used the
30 monoclonal antibody IgG-192 to detect the NGF-receptor.
This monoclonal has been characterized on both rat sensory
neurons and in rat brain (Taniuchi, M. and Johnson, E.,
1985, J. Cell Biol. 101:1100-1106). While confirming the
35 findings of Hartikka and Hefti that NGF can up-regulate the

level of its receptor in septal cultures, as noted by an increase in the number of IgG-192 immunopositive neurons, we have additionally found that BDNF increases the number of IgG-192 immunopositive cells by up to 3 fold. The response to BDNF was biphasic in that high concentrations, in the

5 range of 25-100 ng/ml, failed to produce as large an increase in the number of positive cells as was noted with 10 ng/ml. Interestingly, this type of dose response was quite different from that seen for the BDNF induced increase in the number of AChE positive neurons. In the latter, no

10 reduction in the maximal effect of BDNF was seen at high concentrations. It should be noted that the low-affinity NGF receptor has recently been shown to also bind BDNF with similar affinity, suggesting that the NGF and BDNF low-affinity receptors might be identical (Rodriguez-Tebar et

15 al. 1990, Binding of Brain-Derived Neurotrophic Factor to the Nerve Growth Factor Receptor Neuron. 4, 487-492).

Possible similarities in the mechanism of action of BDNF and NGF were suggested by the findings that BDNF was equipotent with NGF in the induction of AChE activity.

20 Although BDNF was also found to promote cholinergic neuron survival to a similar extent to NGF, the effect of BDNF on CAT enzymatic activity was not as great as NGF. Over several experiments maximal CAT induction with BDNF ranged from 1.8-2.6 fold, while values of greater than 3-fold were

25 seen routinely with NGF. Thus, although there may be some similarities in the mechanism of action of BDNF and NGF, it is quite probable that there are subtle differences in the expression and regulation of their respective receptors and the coupling of these receptors to the second messenger

30 pathway(s) necessary to induce CAT activity.

The supposition that differences in the level of induction of CAT activity produced by BDNF or NGF might represent differential regulatory pathways was further supported by results from time course studies. Cultured

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septal neurons responded to BDNF with a rise in CAT activity
until day 6 at which time the response appeared to plateau.
In contrast, induction in CAT activity in response to NGF
showed a protracted linear rise from 3 to 12 days. The
cessation of a rise in the induction of CAT activity at 6
days in BDNF treated cultures may be indicative of a
developmental change in the expression of either the BDNF
receptor or a required component in the response pathway.
Given that the number of cholinergic neurons (AChE positive
neurons) found in high density cultures after 12 days
appears to be independent of exogenous BDNF or NGF, it seems
unlikely that differential neuronal cell death would account
for the time course differences found in the induction of
CAT activity by these two growth factors.

    In the initial experiments involving BDNF
treatment of basal forebrain cholinergic cells, no
assumption was made as to the primary cell type, neuronal or
glial, responsive to this neurotrophic factor. Although
studies conducted on highly enriched cultures of peripheral
neurons strongly suggest that BDNF has its effects directly
on neurons (Lindsay et al., 1985, Dev. Biol. 112:319-328;
Lindsay, 1988, J. Neurosci. 7:2394-2405), it is conceivable
that the neuronal effects of BDNF seen in the present study
could have been mediated through a primary action of BDNF on
astroglia or other nonneuronal cells. However, BDNF was
found to be equally effective in the induction of CAT
activity in the presence of a confluent monolayer of
predominantly astroglial cells or in cultures greatly
depleted of glial cells by treatment with the mitotic
inhibitor, cytosine arabinoside. An intriguing observation
from these experiments was that the shape of the dose
response curves to BDNF was distinctly different in the two
cases. In the presence of a glial monolayer, the response
to BDNF was linear with an increasing concentration of BDNF.
In neuron-enriched cultures, the dose response to BDNF was

shifted to the left. One possible explanation for these
results is that the glia themselves may express the BDNF
receptor, thus lowering the effective concentration of the
ligand available for the receptor localized on neurons.
Alternatively, the suppressive effect of astrocytes could be
5   indirect, unrelated to an effect on ligand concentration or
receptor expression, and mediated for example by greatly
increased degradation of BDNF. Similar observations have
been reported by Hartikka and Hefti (Hartikka, J. and Hefti,
F., 1988, J. Neurosci. $\underline{8}$:2967-2985) and Honegger and Lenoir
10 (Honegger, P. and Lenoir, D., 1982, Dev. Brain Res. $\underline{3}$:229-
238). It has been demonstrated that glial cells of
different brain regions can greatly influence the morphology
of neurons associated with them (Prochiantz et al., 1979,
Proc. Natl. Acad. Sci. U.S.A. $\underline{76}$:5387-5391; Prochiantz et
15 al., 1981, Nature $\underline{293}$:570-572). Thus, septal glia may have
instrinsic properties either of a membranal or secretory
nature which may act to suppress the expression of the
cholinergic phenotype. The regulatory properties of
astrocytes derived from the hippocampus under similar test
20 conditions is now being investigated.

On the basis of the data presented, it is apparent
that BDNF and NGF are both capable of increasing septal
cholinergic neuronal function. It is interesting to
speculate as to the possible physiological relevance of two
25 highly homologous neurotrophic factors apparently acting on
a single neuronal population. There has been some
suggestion that the actions of BDNF and NGF may initially
overlap during early development of peripheral neurons,
especially on sub-populations of dorsal root ganglion
30 neurons or their precursors, and then later segregate to act
independently on distinct populations (Lindsay et al., 1985,
Dev. Biol. $\underline{112}$:319-328; Ersberger, U. and Rohrer, H., 1988,
Dev. Biol. $\underline{126}$:420-432); Barde, Y.A. 1989, Neuron. $\underline{2}$:1525-
1534). This has not, however, been definitively established
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using appropriate markers to define sub-populations of sensory neurons. Since the present study focused on embryonic septal neurons established in culture at a single time-point in development, E17, it is possible that a similar sort of segregative phenomenon may occur at later development stages in the septum. Thus, there may prove to be interesting temporal and spatial differences in the responsiveness of septal cholinergic neurons to BDNF or NGF.

It will now be interesting to examine the effects of BDNF on basal forebrain cholinergic neurons _in vivo_, where there is now evidence that hippocampal-derived NGF may be involved in the normal development and maintenance of basal forebrain cholinergic neurons _in vivo_. It will be interesting to establish whether or not BDNF has a similar role _in vivo_. The recent finding that BDNF mRNA is particularly abundant in the hippocampus supports such a role.

17.   EXAMPLE: ENZYMATIC CONVERSION OF PREPRO BDNF TO ACTIVE, MATURE BDNF

17.1.   MATERIALS AND METHODS

Commercially available endoproteinase Arg-C (purified from mouse submaxillary glands) was used to enzymatically convert the large precursor form of hBDNF (preproBDNF) to the biologically active mature protein. This enzymatic cleavage was accomplished in an _in vitro_ reaction. The substrate in the enzymatic reaction was preprohBDNF synthesized in CHO-DG44 cells which was secreted into the cell culture media and harvested. The culture media consisted of Ham's F12 media (nucleoside-free) with 1% fetal bovine serum (FBS) and 1% each of penicillin and streptomycin. The reaction was carried out at 37°C for 5 minutes using 5 units of enzyme and 50μl of CHO-DG44 cell supernatant containing prepro hBDNF.

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Cleavage of preprohBDNF to mature hBDNF was complete under these conditions.

17.2.   RESULTS AND DISCUSSION

The prepro form of recombinant hBDNF

5   (approximately 31,000 daltons) was completely processed to the mature bioactive form of hBDNF (approximately 12,000 daltons) in vitro using endoproteinase Arg-C. Recombinant human brain derived neurotrophic factor has been successfully produced in mammalian cells (CHO-DG44 cells).

10 The hBDNF gene has been stably integrated into the CHO cell genome and the hBDNF gene amplified using a methotrexate amplification strategy. The recombinant hBDNF was secreted into the medium and found to be biologically active. Metabolic labeling followed by SDS polyacrylamide gel

15 electrophoresis (15% gel) demonstrates that most of the synthesized [^{35}S]-labeled hBDNF product secreted into the culture medium migrated as the prepro form with an apparent molecular weight of 31,000 (Figure 30, lane 2). [^{35}S]- labeled proteins secreted from wild type CHO-DG44 cells are

20 shown in Figure 30, lane 1. In order to generate predominately the mature form of hBDNF (apparent molecular weight of 12,000), we designed an in vitro strategy to enzymatically cleave the prepro form of hBDNF.

Trypsin digestion products are shown in Figure

25 30, lanes 3-6. Bovine pancreas trypsin (purchased from Worthington; chymotrypsin-free) was dissolved in phosphate buffered saline and added to 50 ul aliquots of [^{35}S]- labeled CHO cell hBDNF. Trypsin concentrations used were 25, 50, 75, and 100 ug/ml. The [^{35}S]-labeled reaction

30 products are shown in lanes 3-6, respectively. The enzymatic cleavage reaction was carried out at 37°C for 5 minutes. A gradual decrease in the labeling intensity of the 31,000 prepro-hBDNF was observed with a corresponding increase in an 18,500 molecular weight product. The mature

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form of hBDNF (molecular weight 12,000) was not generated under these conditions. Figure 30, lane 7 shows the [^{35}S]-labeled reaction products following enzymatic digestion of CHO cell hBDNF with endoproteinase Arg-C isolated from mouse submaxillary gland from Boehringer Mannheim Biochemicals, Inc. 100 units of this enzyme were reconstituted from a lyophilized preparation with 100 ul of Milli-Q water and stored at -20°C. For the enzymatic digestion of CHO cell hBDNF, we used 5 units (5 ul) of enzyme and 50 ul of ^{35}S-labeled protein from CHO cell supernatants containing preprohBDNF (Figure 30, lane 2). As seen in Figure 30, lane 7, 5 units of endoproteinase Arg-C was able to convert the prepro form of hBDNF (31,000 molecular weight) to the mature form of hBDNF (12,000 molecular weight) in 5 minutes at 37°C.

Enzymatic treatment with endoproteinase Arg-C of the supernatants of CHO cells expressing hBDNF increased the level of BDNF biological activity relative to unprocessed supernatants. Supernatants from CHO-DG44 cells expressing hBDNF were treated for 5 minutes with endoproteinase Arg-C at 37°C. 200 ul of supernatant were treated with 20 units of enzyme and both the treated and untreated hBDNF were assayed for biological activity using explants of E8 chick dorsal root ganglia. Twenty-four hours after the addition of hBDNF, neurite outgrowth was qualitatively scored. We consistently observed that the endoproteinase-treated BDNF samples were significantly more bioactive than the control (untreated) hBDNF samples. A concentration curve of this data is plotted in Figure 31.

In conclusion, purified endoproteinase Arg-C can be used in an _in vitro_ enzymatic reaction to generate the mature bioactive form of recombinant human brain derived neurotrophic factor from incompletely processed precursor molecules (i.e., pro BDNF). This novel method will allow for an efficient large scale production of bioactive human

BDNF from mammalian cell culture systems. For example, it
may be possible to immobilize the endoproteinase Arg-C to a
solid matrix and establish an efficient column
chromatography step.

5   18. EXAMPLE: BRAIN-DERIVED NEUROTROPHIC FACTOR
              PROMOTES SURVIVAL AND MATURATION OF DOPAMINERGIC
              NEURONS OF THE RAT VENTRAL MESENCEPHALON

              18.1. MATERIALS AND METHODS

              18.1.1. CELL CULTURES

              Dissociated cultures were established by
10   enzymatic and mechanical dissociation of ventral
mesencephalon tissue dissected from E14-E15 rat embryos.
Typically, pooled tissue from two or three litters of rat
embryos from time-mated Sprague-Dawley rats were
trypsinized (0.125%; Worthington) in F12 medium (Gibco) for
15   20 minutes at 37°C. After washing in growth medium (MEM
containing the following supplements: glutamine, 2mM,
glucose, 6mg/ml, penicillen G, 0.5U/ml, streptomycon
5μg/ml, fetal calf serum, 7.5%) the tissue was briefly
centrifuged at low speed for 5 minutes and the pellet was
20   dissociated by trituration. After allowing 1-2 minutes for
nondispersed cell clumps to settle, the single cell
suspension was seeded onto 35mm dishes (precoated with
polyornithine and laminin; Lindsay et al., 1985, Dev. Biol.
112:319) containing growth medium to give a density of 5 x
25   10^4 cells per cm^2. After an overnight incubation in growth
medium to allow cell attachment, cells were cultured in the
presence or absence of BDNF in a serum-free, defined medium
as described by Bottenstein and Sato (Bottenstein and Sato,
30   1979, Proc. Natl. Acad. Sci. USA 76:514), except that
insulin was included at 20ng/ml. To visualize dopaminergic
cells, cultures were fixed with 4% paraformaldehyde, washed
extensively, permeabilized with 0.02% Saponin in Sorensen's
buffer with 1.5% horse serum and stained with a mouse

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monoclonal antibody to rat TH (Boehringer-Mannheim).
Primary antibody binding was visualized using a Vectastain
ABC kit (Vector Labs).

18.1.2. MEASUREMENT OF DOPAMINE UPTAKE

Cultures were prepared as described in 18.1.1.,
supra, and were grown for the number of days indicated,
either in the presence or absence of porcine BDNF, 50
ng/ml. Dopamine uptake was determined in triplicate
cultures at the time points indicated. Cells were
prewashed in uptake-buffer of the folowing composition:
136.8 mM NaCl, 2.7 mM KCl, 8mM $Na_2HPO_47H_2O$, 1.5mM KH_2PO_4,
5mM glucose, 1mM $CaCl_2$, 1mM $MgSO_4$, 0.1mM ascorbate and
0.1mM pargyline, pH 7.4. Those samples to be measured for
nonneuronal ^{3}H-dopamine uptake had benztropine included
(BZT) in the uptake buffer, at a concentration of 5 μM.
After washing, the cells were prewarmed to 37°C for five
minutes in fresh uptake buffer at which point ^{3}H-dopamine
(NEN, 40 Ci/mmol) was added to a final concentration of
50nM. The cultures were incubated at 37°C for 15 minutes,
the uptake solution was removed and the cells were placed
on ice, and washed four times with ice-cold uptake buffer.
Cells were harvested by addition of 0.5N NaOH to the
culture dishes, and the NaOH extract was counted in a
Beckman scintillation counter with 15 mls Ultima Gold
scintillation fluid (Packard Instruments). Specific
neuronal dopamine uptake is defined as that uptake (cpm)
observed in the absence of BZT less that observed in the
presence of BZT. Routinely, between 70 - 90% of the total
uptake was inhibitable by BZT. In replicate cultures at
each time point the number of TH+ neurons was determined by
immunocytochemical staining and the results shown represent
uptake normalized on a per TH+ basis.

18.1.3. TRANSIENT EXPRESSION OF BDNF

Cos M5 cells were transfected with the vector CDM8 containing the sequence encoding human BNDF. At 72 hours post transfection, 50ml of culture supernatant was collected and dialyzed against 6M urea. The dialyzed

5 supernatant was then combined with ampholines (pH 3.5 - 10, BioRad) for 4 1/2 hours. Fractions were collected, dialyzed against 25mM $NaPO_4$ buffer pH 7.6 using dialysis tubing which had been prewashed in BSA (0.5mg/ml) to prevent nonspecific absorbtion. Fractions were assayed for

10 neurite outgrowth promoting activity in cultures of E8 chick dorsal root ganglion explants. Active fractions were pooled. Analysis of the active pool by SDS PAGE and subsequent silver staining (Wray et al., 1981, Anal. Biochem. 118:197) on an 8-18% gel revealed a faint band at

15 68 kD corresponding to BSA, and a single band at approximately 12kD, corresponding to that observed previously for porcine BDNF (Barde et al., 1982, EMBO J. 1:549) (data not shown). In comparative bioassays carried out on chick dorsal root, nodose and sympathetic ganglion

20 explants the specific activity and neuronal specificity of purified recombinant human BDNF was similar to that of purified porcine BDNF.

18.1.4. PRODUCTION OF hBDNF FROM TRANSFECTED CHO CELLS

25   CHO-DG44 cells were a kind gift of Dr. L. Chasin (Columbia University, NY). These cells are deficient in dihydrofolate reductase (dhfr-) (Urlaub and Chasin, 1980, Proc. Natl. Acad. Sci. USA 77:4216-4220). CHO-DG44 cells were maintained in Ham's F12 medium with 10% fetal bovine

30 serum, 1% penicillin and streptomycin, and 2mM glutamine. Cells were passaged twice a week and were routinely checked for mycoplasma contamination. All plasmid DNA was purified by double cesium chloride banding prior to transfection. The human brain-derived neurotrophic factor gene was

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subcloned into the mammalian expression vector pCDM8 to generate pC8hB as described previously (Maisonpierre et al., 1990, Science 247:1446-1451). A weakened dihydrofolate reductase gene, p410, was kindly provided by Dr. L. Chasin. These two gene constructs were

5 cotransfected into CHO-DG44 cells by electroporation (reviewed in Shigekawa and Dower, 1988, BioTechniques 6:742-751). Approximately 1×10^6 cells were transfected with 20ug of pC8hB and 0.2ug of p410. 48 hours after transfection, the CHO-DG44 cells were split into selection

10 media consisting of nucleoside-free Ham's F12 (without thymidine and hypoxanthine; -HT) plus 10% dialyzed fetal bovine serum and 1% penicillin and streptomycin. Individual colonies were isolated approximately 10 days after placing the cells in nucleoside-free selection

15 medium. Individually selected colonies resistant to growth in necleoside-free Ham's F12 (-HT) medium were treated with either 0.02, 0.05, or 0.1uM methotrexate (MTX) to induce gene amplification (Alt et al., 1978, J. Biol. Chem. 253:1357-1370). MTX-resistant colonies were cultured as

20 pools and were further amplified with either 1.5, 2.0, or 2.5uM MTX. A single colony resistant to 2.5uM MTX was isolated and selected for scale-up culture and production of hBDNF. Southern blotting analysis of this clone (DGZ1000-B-3-2.5) indicated an amplification of the BDNF

25 gene relative to wild-type CHO-DG44 cells of approximately 50-fold. Bioassays with explants of embryonic (E8) chick dorsal root ganglia (DRG) were used to estimate that this clone was producing approximately 9.5ug of hBDNF/ml (not shown). Recombinant hBDNF was produced on a large scale by

30 culturing DGZ1000-B-3-2.5 cells in 480 cm^2 roller bottles. Media was harvested approximately four days after these cells reached confluence. Purification of hBDNF from culture supernatant was performed as above for COS

35 supernatant.

18.2. RESULTS AND DISCUSSION

Characterization of molecular signals which control neuronal survival and specificity of target innervation is of fundamental interest, not only because it will help to elucidate how the nervous system is shaped but

5 also because it is likely to lead to a better understanding of the mechanisms involved in maintenance and repair of mature neurons in appropriate synaptic configurations. While it is widely recognized that the survival and maintenance of neurons depend upon specific, target cell-

10 derived neurotrophic factors (Levi-Montalcini and Angeletti, 1968, Physiol. Rev. 48:534; Thoenen and Barde, 1980, Physiol. Rev., 60:1284; Purves, D., 1988, in Body and Brain, Harvard University Press, Cambridge, MA.; Barde, Y.A., 1989, Neuron, 2:1525; Lindsay, R.M. 1988, The Making

15 of the Nervous System, Oxford University Press, Oxford, p. 148) very few such molecules have been fully characterized. To date, only two neurotrophic factors, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), have been shown to selectively support the survival of distinct

20 neuronal populations in vivo (Levi-Montalcini and Angeletti, 1968, Physiol. Rev. 48:534; Barde, Y.A., 1989, Neuron, 2:1525; Leibrock et al., 1989, Nature 341:149).

Neuronal cell culture has been widely used to establish bioassays for the identification of neurotrophic

25 activity in cell and tissue extracts (Levi-Montalcini and Angeletti, 1968, Physiol. Rev. 48:534; Thoenen and Barde, 1980, Physiol. Rev., 60:1284; Lindsay, R.M. 1988, The Making of the Nervous System, Oxford University Press, Oxford, p. 148; Varon and Adler, 1981, Adv. Cell.

30 Neurobiol., 2:118) and to determine the neuronal specificity(ies) of purified neurotrophic factors (Ebendal, T., 1989, in Nerve Growth Factors, John Wiley, London, p. 81; Barbin et al., 1984, J. Neurochem. 43:1468; Barde et al., 1982, EMBO J. 1:549; Davies et al., 1986, J. Neurosci.

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6:1897; Lindsay et al., 1985, Dev. Biol. 112:319). To
date, most studies have concentrated on the neurotrophic
requirements of different classes of neurons of the
peripheral nervous system (PNS). The availability of NGF
has established it as by far the most well characterized
5 neurotrophic factor: NGF has been shown both in vitro and
in vivo to be essential for the survival and maintenance of
neuronal subpopulations of both the PNS and CNS (Levi-
Montalcini and Angeletti, 1968, Physiol. Rev. 48:534;
Thoenen and Barde, 1980, Physiol. Rev., 60:1284; Whittemore
10 and Seiger, 1987, Brain Res. Rev. 12:439; Snider and
Johnson, 1989, Ann. Neurol. 26:489; Hefti et al., 1989,
Neurobiology of Aging 10:515; Martinez et al., 1987, Brain
Res. 412:295; Takei et al., 1988, J. Neurochem. 51:1118;
Hartikka and Hefti, 1988, J. Neurosci. 8:2967). Hampering
15 the identification of neurotrophic growth factors for
specific CNS neurons has been the limited availability of
purified preparations of neurtrophic factors other than
NGF, and the difficulty in preparing homogeneous
preparations of specific neuronal populations. Two recent
20 observations prompted us to study the effects of BDNF on
the survival of CNS (nigral) dopaminergic neurons.
Firstly, it is now clear that the BDNF gene is expressed in
the CNS, and at comparably or higher levels than the NGF
gene (Leibrock et al., 1989, Nature 341:149). Secondly, it
25 has been reported that a protein partially purified from
bovine striatum (a target of nigral dopaminergic neurons),
with characteristics apparently similar to those of BDNF,
can increase the in vitro survival of dopaminergic neurons
prepared from the mesencephalon (Dal Toso et al., 1988, J.
30 Neurosci 8:733).

Figure 32A illustrates the typical appearance of
dissociated ventral mesencephalic cells after nine days in
culture. Virtually all of the cells had phase bright
35 perikarya and long processes. Few cells of fibroblast

morphology were evident and no astroglial cells were
detected when cultures were stained with antibodies for the
astroglial marker, glial fibrillary acidic protein (GFAP;
Bignami et al., 1972 Brain Res. 43:429). To visualize
dopaminergic neurons in these culture, immunocytochemical
5   staining was carried out with monoclonal antibodies to TH.
As expected, the number of TH+ neurons was small (arrows,
Fig. 32B, 32C) and varied, depending on the time in
culture, between 0.1-0.5% of the cells plated. Various
neuronal morphologies were noted among the TH+ cells, as
10   shown in Figure 33.

In all cultures, there was invariably a gradual
decline in the number of TH+ cells (Figs. 34A, 35) after
the first 3-4 days in vitro. By 8 days in culture, for
example, the number of TH+ cells in control cultures was
15   only 25% of that found in similar cultures at day 2 (Fig.
35). However, in cultures treated with BDNF, the loss of
TH+ cells was greatly reduced when compared to controls.
At all times examined after 8 days _in vitro_ the number of
TH+ cells in BDNF treated cultures was greater than in the
20   untreated controls, e.g. 1.8-fold higher after a single
addition of BDNF (Fig. 34A) and 5-fold higher after
multiple additions (Fig. 35). Even when added only once to
cultures maintained for 8 days, the effect of BDNF was
found to be dose dependent (Fig. 34B). In agreement with
25   previous reports (Dal Toso et al., 1988, J. Neurosci 8:733;
Knusel et al. 1990, J. Neuroscii. 10:558), NGF (50 ng/ml)
had no effect on the number of TH+ cells (Fig. 34C).

As a further way of examining the effect of BDNF
on the dopaminergic neurons in these cultures, the capacity
30   of the TH+ cells to take up ^{3}H-dopamine was studied. As
shown in Table X, dopamine uptake capacity (normalized per
TH+ neuron) increased markedly over the first 8 days in
culture but dropped thereafter. It was found, however,
that BDNF did not change the capacity of TH+ cells to take
35

up dopamine, as the same time course and similar values were obtained in the presence or absence of BDNF (Table X). We hypothesized from this result that BDNF probably acts directly to promote survival of dopaminergic neurons in mesencephalic cultures as opposed to inducing the

5   expression of a dopaminergic phenotype in neurons which do not necessarily require BDNF for survival. To examine this further, we carried out experiments in which the number of TH+ neurons was determined in culture in which BDNF addition was initially delayed for several days. We found

10   that when BDNF addition was delayed until day 5, and the number of TH+ cells was subsequently determined at 6, 8 or 10 days, fewer dopaminergic cells were seen in these cultures when compared to parallel cultures in which BDNF had been present from day 2 onwards (Fig. 36). Although

15   delayed addition of BDNF clearly increased the number of TH+ neurons compared to controls, when examined even at equivalent time points the effect of delayed addition was never found to rescue as many TH+ neurons as addition of BDNF on day 2.

20   Taken together, these results argue against the possibility that BDNF acts only to increase TH-gene expression within a fixed number of cells, and suggest that BDNF exerts its effect by increasing survival of dopaminergic neurons that are otherwise lost when this

25   neurotrophic factor is not added to cultures at an early stage.

           Several reports have documented the stimulation of maturation and enhancement of survival in vitro of

30   mesencephalic dopaminergic neurons by either target-derived extracts or various growth factors (Dal Toso et al., 1988, J. Neurosci. 8:733; Knusel et al. 1990, J. Neurosci. 10:558; Prochiantz et al., 1979, Proc. Natl. Acad. Sci. USA 76:5387; DiPorzio et al., 1980, Nature 288:370; Prochiantz

35   et al., 1981, Nature 293:570; Denis-Donini et al., 1983, J.

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Neurosci. 3:2292). So far, however, there has been no
report of a fully purified molecule directly and
selectively supporting the survival of TH+ neurons in the
complete absence of glial cells or enhanced cell division
(as observed, for example, with IGF-1 or FGF, Dal Toso et

5   al., 1988, J. Neurosci 8:733). In agreement with previous
reports using early embryonic mouse tissue (Dal Toso et
al., 1988, J. Neurosci 8:733), E14 rat ventral
mesencephalic cells cultured in serum-free, defined medium
were found to be essentially free of astrocytes or

10 fibroblasts. The relatively low cell plating density of
50,000 cells/cm^2 used in this study diminished the effects
of any possible endogenous neurotrophic activity, and
allowed for more accurate cell counting. In view of the
results presented here, it appears that the neurotrophic

15 factor, partially purified from bovine striatum by Del Toso
et al. (Dal Toso et al., 1988, J. Neurosci 8:733), is
likely to be BDNF. This suggests that BDNF is produced in
the striatum and taken up by the terminals of the
dopaminergic neurons projecting from the substantia nigra.

20 As yet, however, mRNA for BNDF (or indeed mRNA for other
members of the neurotrophin family) has not been found to
be abundant in striatal tissue by Northern blot analyses.

        It is clear that BDNF addition, even in the case
of multiple additions, does not result in complete survival

25 of nigral dopaminergic neurons during the culture periods
analyzed. This phenomenon has already been observed under
experimental conditions very similar to ours by Dal Toso et
al. (Dal Toso et al., 1988, J. Neurosci 8:733) who showed
that this loss might be related to the cell density used;

30 at higher cell densities (4 times that used here) a larger
number of catecholamine-fluorescent cells could be seen and
the spontaneous disappearance of these cells was decreased.
Possibly, cell-cell contact is needed for long term

35 survival of these cells.

Further studies, particularly those directed at examining the effects of BDNF on the development and maintenance of dopaminergic neurons of the ventral mesencephalon in vivo, will be necessary to determine the physiological relevance of the effects of BDNF described here. In view of the specific loss of nigral dopaminergic neurons in Parkinson's disease, it is particularly desirable to find a neurotrophic factor which selectively affects these cells. Thus, the present finding that BDNF supports the survival of these neurons, cells which are refractory to NGF, provides rationale for animal experiments which will determine whether BDNF can protect dopaminergic neurons from the neurotoxic effects of either 6-hydroxydopamine or MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). Such studies may lead to a novel therapeutic approach for Parkinsonism.

TABLE X

DOPAMINE UPTAKE IN VENTRAL MESENCEPHALIC
CULTURES IS NOT INCREASED DIRECTLY BY BDNF

^{3}H-Dopamine Uptake

(cpm/TH /(+) neuron/15 minutes)

Days In Culture	Control	BDNF-Treated
3	0.51 ± 0.1	0.95 ± 0.2
6	4.5 ± 0.3	3.1 ± 0.6
8	18.5 ± 5.3	27.3 ± 1.2
10	3.37 ± 0.24	2.21 ± 0.18

---

19. EXAMPLE: BDNF PRODUCES A DOSE-DEPENDENT
     INHIBITION OF GAMMA-AMINOBUTYRIC ACID UPTAKE
     19.1. MATERIALS AND METHODS

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19.1.1. HIPPOCAMPAL CELL CULTURES

Hippocampi were dissected from E18-19 rat embryos of Sprague-Dawley rats, and collected in F10 medium. The tissues were minced, rinsed twice with F10 medium (Gibco) and trypsinized with 0.25% trypsin (Gibco)

5   for 20 minutes at 37°C. Trypsin was inactivated by the addition of a serum-containing medium composed of minimum essential medium (MEM) supplemented with fetal calf serum. (FCS, 10%), glutamine (2mM), pencillin (25U/ml) and streptomycin (25ug/ml). Dissociated cells obtained by

10 gentle trituration were collected and centrifuged at low speed (500 rpm) for 30 seconds. The centrifugation was repeated twice, and the cell pellets were then resuspended in serum-containing medium. The cells were then plated onto 6 mm wells or 35 mm dishes that were coated with

15 polyornithine (10ug/ml) and laminin (10ug/ml). In most of the experiments, the cells were plated at a low density of approximately 71,000 cells/cm^2. Five to six hours following the plating of cells, medium was changed to a serum-free medium containing 1% N3 and penicillin-

20 streptomycin (25 units/ml and 25 ug/ml, respectively), at which time BDNF was added. Medium was changed every three to four days, with re-addition of the factor.

19.1.2. MEASUREMENT OF HIGH AFFINITY UPTAKE FOR GAMMA-AMINOBUTYRIC ACID (GABA)

25   High-affinity GABA uptake was measured using a modified procedure of Tomozawa and Appel (1986, Brain Res. 399:111-124). Cells were washed in the GABA uptake buffer containing 140mM NaCl, 2.6 mM KCl, 1 mM KH_2PO_4, 1 mM

30 Na_2HPO_4, 6 mg/ml glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% BSA. Following washing, cells were incubated with the GABA uptake buffer for 5 minutes at 37°C. ^{3}H-GABA (NEN, NET-191X, 111.4 Ci/mmol) was then added at a final concentration of 12 nM, and incubation was carried out at

35

37°C for 10 minutes. Cells were then kept on ice, and washed three times with the uptake buffer. Cells were incubated with 0.14 N NaOH for 2 hours at room temperature, and ^{3}H-GABA in the extract was counted. ^{3}H-GABA uptake was found to be linear for up to at least 30 minutes. Uptake

5 of GABA into non-neuronal cells was inhibited by the addition of 2 mM B-alanine, whereas uptake specific for neurons is verified by inhibition with nipecotic acid at 1 mM.

10   19.2. RESULTS AND DISCUSSION

We have recently detected abundant levels of message for BDNF in neuron-enriched hippocampal cultures, but not in hippocampal astrocytes. These data strongly suggest that BDNF is localized to hippocampal neurons. In

15 order to examine the effect of BDNF on hippocampal neurons in culture, cells were treated with various concentrations of BDNF (rotorphor-purified from COS supernatants). At the end of the 8 days treatment period, high affinity GABA uptake into neurons was measured. As shown in Figure 37,

20 BDNF produced a dose-dependent inhibition of GABA uptake. Thus, BDNF may affect the survival and/or phenotypic expression of GABAergic neurons. BDNF did not have any effect on glutamate-containing neurons (as assessed by glutamate-uptake measurements), nor on the levels of

25 neurofilament protein. Thus, the effect of BDNF on hippocampal cultures appears to be specific for Gabaergic neurons.

30   20. EXAMPLE: BDNF CONFERS A PROTECTIVE
                 EFFECT AGAINST TOXIC EFFECTS OF MPP+
                20.1. MATERIALS AND METHODS
         20.1.1. MEASUREMENT OF THE EFFECTS OF NEUROTROPHIC
                 FACTORS ON MPP+-TREATED SH-SY5Y CELLS

35

SH-SY5Y cells were seeded into 24 well plates at
a density of 1 x 10^5 cells per well. Twenty four hours
after seeding, the cells were treated with neurotrophic
factors. Twenty four hours after neurotrophic factor
treatment, the cells were treated with MPP+ (10 uM).
5 Forty-eight hours after MPP+ addition, viable cells were
quantitated by the trypan blue exclusion assay.
Neurotrophic factors utilized were mNGF-COS (1:5 dilution),
hBDNF-COS (1:5 dilution), purified rCNTF from E. coli (25
ng/ml), purified bovine brain bFGF (25 ng/ml), rNT3-COS
10 (1:5 dilution), and purified hEGF (25 ng/ml).

20.1.2. MEASUREMENT OF THE EFFECTS OF NEUROTROPHIC FACTORS ON MPP+-TREATED VENTRAL MESENCEPHALIC CULTURES

15   Cultures of dissociated ventral mesencephalic
neurons were established from E14 rat embryos according to
established protocols (See Section 18, supra). Forty eight
hours after these cultures were established, they were
treated with the appropriate neurotrophic agents as
indicated. Neurotrophic factors included hBDNF (rotophor-
20 purified from CHO cells, ≤50 ng/ml), purified mNGF (50
ng/ml), rNT-3 (COS supernatants at 1:50 dilution), purified
bovine brain bFGF (10 ng/ml), and purified rCNTF (25 ng/ml)
from E. coli). Twenty four hours after neurotrophic factor
addition, these cultures were treated with MPP+ (1 uM).
25 Forty eight hours after MPP+ treatment, TH positive neurons
were identified by immunohistochemistry and quantitated.
Data represent the mean of three independent experiments
done with triplicate samples per experiment. The number of
TH-positive neurons following MPP+ treatment is represented
30 by the cross-hatched bars. Open bars represent the number
of TH-positive neurons without MPP+ treatment.

35

20.2. RESULTS AND DISCUSSION

When BDNF, NGF, CNTF, NT-3, bFGF, and EGF were separately tested for the ability to protect SH-SY5Y cells from 1-methyl-4-phenyl pyridinium (MPP+) toxicity, as measured by trypan blue exclusion assay, only BDNF and NGF appeared to exhibit significant protective activity against MPP+ toxicity (Tables XI and XII).

TABLE XI

BDNF AND NGF PROTECTION OF SH-SY5Y CELLS FROM MPP+ TOXICITY

Results:

Treatment:	Number of Viable Cells (% Viable)
COS-Mock transfected (1:5)	0.4×10^4 (5%)
BDNF-COS (1:5)	1.2×10^5 (67%)
NGF-COS (1:5)	1.7×10^5 (84%)
CNTF	0.6×10^4 (14%)
NT-3	0.7×10^4 (20%)
bFGF	0.4×10^4 (11%)
EGF	0.1×10^4 (3%)

TABLE XII

BDNF AND NGF CONCENTRATION CURVE
FOR MPP+ TOXICITY EXPERIMENTS

Treatment		% Viable Cells
COS-MOCK	(1:2)	13
	(1:5)	5
	(1:10)	3
	(1:20)	6
	(1:50)	7
	(1:100)	2
COS-BDNF	(1:2)	73
	(1:5)	67
	(1:10)	65
	(1:20)	42
	(1:50)	30
	(1:100)	25
COS-NGF	(1:2)	88
	(1:5)	84
	(1:10)	63
	(1:20)	50
	(1:50)	47
	(1:100)	34

In ventral mesencephalic cultures, the data indicate that BDNF exhibited a significant protective effect against MPP+ toxicity, whereas bFGF exerted a lesser effect (Figure 38). MPP+ treatment was found to reduce the number of tyrosine hydroxylase positive neurons by 85% in control cultures but only by 40% in cultures pretreated

with BDNF prior to MPP+ exposure. MPP+ is the presumed
active metabolite for the toxin MPTP, which has been
observed to induce a Parkinson's disease-like syndrome in
vivo, which is an accepted model system for Parkinson's
disease. Thus, the protective effect of BDNF and NT-3
indicate that these compounds may prove useful in the
treatment of Parkinson's disease or in the prevention of
neurologic damage subsequent to toxic exposure.

21. DEPOSIT OF MICROORGANISMS

The following recombinant bacteriophage and
recombinant plasmid DNA were deposited on August 30, 1989,
with the American Type Culture Collection, 12301 Parklawn
Drive, Rockville, Maryland 20852:

Accession No.	ATCC
phBDNF-C-1	40648
λhBDNF-G-1	40649

The present invention is not to be limited in scope
by the specific embodiments described herein. Indeed,
various modifications of the invention in addition to those
described herein will become apparent to those skilled in
the art from the foregoing description and accompanying
figures. Such modifications are intended to fall within
the scope of the appended claims.

Various publications are cited herein, the
disclosures of which are incorporated by reference in their
entireties.

International Application No: PCT/   /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page __149__ , line __19-20__ of the description [1]

A. IDENTIFICATION OF DEPOSIT [1]

Further deposits are identified on an additional sheet [X] [1]

Name of depositary institution [1]

American Type Culture Collection

Address of depositary institution (including postal code and country) [1]

12301 Parklawn Drive
Rockville, MD   20852, US

Date of deposit [1]	Accession Number [1]
September 7, 1989	40648

B. ADDITIONAL INDICATIONS [1] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [1] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [1] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [1] (Specify the general nature of the indications e.g. "Accession Number of Deposit")

E. [ ] This sheet was received with the International application when filed (to be checked by the receiving Office)

_____
(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau [1]

_____
(Authorized Officer)

Form PCT/RO/134 (January 1981)

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Page 2

MICROORGANISMS – cont'd

American Type Culture Collection

12301 Parklawn Drive
Rockville, MD 20852
US

Date of Deposit: September 7, 1989   Accession Number 40649

WHAT IS CLAIMED IS:

1. A recombinant DNA molecule comprising a nucleic acid sequence encoding brain derived neurotrophic factor (BDNF) or a subsequence thereof comprising about at least 10 nucleotides.

5

2. The recombinant DNA molecule of claim 1 in which the brain derived neurotrophic factor encoding nucleic acid sequence or subsequence is substantially as depicted in FIG. 1 from about nucleotide 167 to about nucleotide 927.

10

3. The recombinant DNA molecule of claim 1 or 2 which comprises a cDNA sequence.

4. The recombinant DNA molecule of claim 1 or 2 which comprises a genomic DNA sequence.

15

5. The recombinant DNA molecule of claim 1 which comprises a human DNA sequence or subsequence substantially as depicted in Figure 5.

20

6. The recombinant DNA molecule of claim 5 which comprises at least a portion of the sequence substantially as depicted in Figure 5 between about nucleoside 974 and nucleoside 1440 nucelotides.

25

7. The recombinant DNA molecule of claim 1 or 2 which comprises a porcine DNA sequence.

8. The recombinant DNA molecule of claim 5 as contained in phBDNF-C-1 deposited with the ATCC and with the accession number 40648.

30

9. The recombinant DNA molecule of claim 5 as contained in λhBDNF-G-1 deposited with the ATCC and with the accession number 40649.

35

10.   A recombinant ribonucleic acid molecule
complementary to the recombinant DNA molecule of claim 1 or
2.

5

11.   A nucleic acid sequence comprising a sequence
encoding a protein substantially homologous to the amino acid
sequence depicted in Figure 1, or portions thereof.

10

12.   A nucleic acid sequence comprising a sequence
encoding a protein substantially homologous to the amino acid
sequence depicted in Figure 5, or portions thereof.

13.   A nucleic acid sequence comprising a sequence
substantially homologous to the nucleic acid sequence

15

depicted in Figure 1, or a hybridizable portion thereof.

14.   The recombinant DNA molecule of claim 1 or 2 in
which the expression of nucleic acid sequence encoding BDNF
protein or peptide fragment is regulated by a second nucleic

20

acid sequence so that brain derived neurotrophic factor
protein or peptide is expressed in a host transformed with
the recombinant DNA molecule.

25

15.   The recombinant DNA molecule of claim 14 as
contained in pCMV-pBDNF-1.

16.   A nucleic acid vector comprising the DNA
molecule of claim 1.

30

17.   A recombinant microorganism containing the DNA
molecule of claim 1, 2, or 13.

18.   The recombinant microorganism of claim 17 which

35   is a bacterium.

19.   The recombinant microorganism of claim 18 which is a yeast.

20.   A cell containing the recombinant DNA molecule of claim 13.

21.   A cell comprising the recombinant DNA molecule of claim 14.

22.   A nucleic acid sequence comprising a sequence encoding the amino acid sequence substantially as depicted in Figure 1 or a sequence thereof comprising an antigenic determinant.

23.   A purified protein having an amino acid sequence substantially as depicted in Figure 1 or a subsequence thereof comprising an antigenic determinant.

24.   A purified protein having an amino acid sequence substantially as depicted in Figure 1 or a subsequence thereof comprising a functionally active peptide.

25.   A purified protein having an amino acid sequence substantially as depicted in Figure 5 or a subsequence thereof comprising an antigenic determinant.

26.   A purified protein having an amino acid sequence substantially as depicted for human BDNF in Figure 5 or a subsequence thereof comprising a functionally active peptide.

27. A purified protein having an amino acid
sequence substantially as depicted in Figure 1, comprising
from about amino acid 134 to about amino acid 252, or a
subsequence thereof comprising an antigenic determinant.

5

28. A purified protein having an amino acid
sequence encoded by the nucleic acid sequence substantially
as depicted for chicken BDNF in Figure 5 or a subsequence
thereof comprising an antigenic determinant.

10

29. A purified protein having an amino acid
sequence encoded by the nucleic acid sequence substantially
as depicted for chicken BDNF in Figure 5 or a subsequence
thereof comprising a functionally active peptide.

15

30. A purified protein having an amino acid
sequence encoded by the nucleic acid sequence substantially
as depicted for rat BDNF in Figure 5 or a subsequence thereof
comprising an antigenic determinant.

20

31. A purified protein having an amino acid
sequence encoded by the nucleic acid sequence substantially
as depicted for chicken BDNF in Figure 5 or a subsequence
thereof comprising a functionally active peptide.

25

32. A method for producing BDNF protein or a
fragment thereof comprising growing a recombinant
microorganism containing the DNA molecule of claim 1, such
that the DNA molecule is expressed by the microorganism; and
isolating the expressed BDNF protein or fragment.

30

35

33. A method for producing BDNF protein or a
fragment thereof comprising growing a recombinant
microorganism containing the DNA molecule of claim 2, such
that the DNA molecule is expressed by the microorganism; and
isolating the expressed BDNF protein or fragment.

34. A method for producing BDNF protein or a
fragment thereof comprising (a) growing a recombinant
microorganism containing the DNA molecule of claim 14, under
conditions such that the DNA molecule is expressed by such
microorganism; and (b) isolating the expressed BDNF protein
or fragment.

35. A method for producing BDNF protein or a
fragment thereof comprising (a) growing a recombinant
microorganism containing the DNA molecule of claim 15, under
conditions such that the DNA molecule is expressed by the
microorganism; and (b) isolating the expressed BDNF protein
or fragment.

36. A method for producing BDNF protein or a
fragment thereof comprising (a) growing a recombinant host
containing the DNA molecule of claim 2, under conditions such
that the DNA molecule is expressed by the host; and then (b)
isolating the expressed BDNF protein or fragment.

37. The method according to claim 36 in which the
host is a eukaryotic cell.

38. The method according to claim 37 in which the
host is non-human transgenic animal.

39.   The product of the method of claim 32.

40.   The product of the method of claim 33.

41.   The product of the method of claim 34.

42.   The product of the method of claim 35.

43.   The product of claim 39 which is free of detergent.

44.   The product of claim 40 which is free of detergent.

45.   The product of claim 41 which is free of detergent.

46.   The product of claim 42 which is free of detergent.

47.   The product of claim 36, 39, or 40 which is glycosylated.

48.   The product of claim 36, 39, or 40 which is unglycosylated.

49.   An antibody, antibody fragment, or derivative thereof which recognizes a BDNF protein, peptide fragment or derivative.

50.   The antibody, antibody fragment, or derivative of claim 49 which is produced by a method comprising immunizing a host animal with a BDNF protein, peptide fragment or derivative, which BDNF protein fragment or

derivative is produced by growing a recombinant organism
expressing a recombinant nucleic acid vector encoding the
BDNF protein, fragment, or derivative.

5   51.   The antibody, antibody fragment, or derivative
of claim 49 in which the nucleic acid vector comprises a
nucleic aid sequence substantially as depicted in Figure 1,
from about nucleotide 167 to about nucleotide 927, or a
subsequence thereof.

10

      52.   The antibody, antibody fragment, or derivative
of claim 49 which is produced by a method comprising
immunizing a host animal with the purified BDNF protein of
claim 23, or a peptide fragment or derivative thereof.

15

      53.   The antibody, antibody fragment, or derivative
of claim 49 which recognizes the B5 peptide fragment
comprising residues 153 to 185 of the amino acid sequence
substantially as depicted in Figure 1.

20

      54.   A recombinant DNA molecule which encodes a
protein or peptide which comprises (a) a first amino acid
sequence homologous to two different known members of the
BDNF/NGF gene family, and (b) a second amino acid sequence
25   which is not homologous to either BDNF or NGF.

      55.   The recombinant DNA molecule of claim 54 in
which one known member is BDNF.

30

      56.   The recombinant DNA molecule of claim 54 in
which one known member is NGF.

      57.   A recombinant DNA molecule of claim 54 which
35   is isolated by a method comprising

(a)   selecting, from among a diversity of nucleic acid sequences, those sequences that are homologous to both BDNF and NGF; and

(b)   identifying, from among the nucleic acid sequences selected in (a), those sequences which contain sequences of about at least 6 contiguous nucleotides that are not homologous to NGF and BDNF.

58.   The recombinant DNA molecule of claim 57 in which the method of step (a) comprises using the polymerase chain reaction technique, in which a pair of oligonucleotide primers are used which are capable of hybridizing to homologous regions of both BDNF and NGF DNAs, such that the sense strand primer is capable of hybridizing to a first homologous DNA sequence of BDNF and NGF, and the antisense strand oligonucleotide primer is capable of hybridizing to a second homologous DNA sequence of BDNF and NGF.

59.   The recombinant DNA molecule of claim 58 in which the sense strand oligonucleotide primer comprises a nucleic acid sequence substantially as depicted in Figure 1, which is selected from the group consisting of and the antisense strand oligonucleotide primer comprises a nucleic acid sequence substantially as depicted in Figure 1, which is (a) selected from the group consisting of from about residue 587 to about residue 616, from about residue 713 to about residue 739, from about residue 764 to about residue 781, from about residue 863 to about residue 898, and subsequences thereof; and (b) is different from the sense strand primer.

60. The recombinant DNA molecule of claim 54 which is homologous to the BDNF nucleotide sequence as depicted in Figure 1 from about residue 587 to about residue 616 or a corresponding nucleotide sequence in a BDNF gene from another species.

61. The recombinant DNA molecule of claim 54 which is homologous to the BDNF nucleic acid sequence as depicted in Figure 1 from about residue 713 to about residue 739 or a corresponding nucleotide sequence in a BDNF gene from another species.

62. The recombinant DNA molecule of claim 54 which is homologous to the BDNF nucleic acid sequence as depicted in Figure 1 from about residue 764 to about residue 781 or a corresponding nucleotide sequence in a BDNF gene from another species.

63. The recombinant DNA molecule of claim 54 which is homologous to the BDNF nucleic acid sequence as depicted in Figure 1 from about residue 863 to about residue 898 or a corresponding nucleotide sequence in a BDNF gene from another species.

64. A pharmaceutical composition comprising an effective amount of substantially pure BDNF protein in a pharmaceutically suitable carrier.

65. A pharmaceutical composition comprising an effective amount of a substantially pure, functionally active BDNF peptide fragment or derivative in a pharmaceutically suitable carrier.

66. A pharmaceutical composition comprising an effective amount of a substantially pure BDNF peptide fragment or derivative carrying an antigenic determinant, in a pharmaceutically suitable carrier.

67. The pharmaceutical composition of claim 65 or 66 in which the BDNF peptide fragment or derivative comprises at least a portion of the amino acid sequence substantially as depicted in Figure 1.

68. The pharmaceutical composition of claim 64 in which the BDNF protein comprises the amino acid sequence substantially as depicted in Figure 1.

69. The pharmaceutical composition of claim 64 in which the BDNF protein comprises the amino acid sequence substantially as depicted in Figure 1 from about amino acid 134 to about amino acid 252.

70. A pharmaceutical composition comprising an effective amount of the product of claim 36, 39, or 40 in a pharmaceutically suitable carrier.

71. A pharmaceutical composition comprising an effective amount of the product of claim 43 or 44 in a pharmaceutically suitable carrier.

72. A pharmaceutical composition comprising an effective amount of the product of claim 45 in a pharmaceutically suitable carrier.

73. A pharmaceutical composition comprising an effective amount of the product of claim 46 in a pharmaceutically suitable carrier.

74.   A pharmaceutical composition comprising an effective amount of the product of claim 47 in a pharmaceutically suitable carrier.

75.   A pharmaceutical composition comprising an effective amount of the product of claim 48 in a pharmaceutically suitable carrier.

76.   The pharmaceutical composition of claim 68 or 69 which is capable of increasing neuron survival.

77.   The pharmaceutical composition of claim 68 or 69 which is capable of increasing neuron growth.

78.   The pharmaceutical composition of claim 68 or 69 which is capable of supporting differentiated cell function.

79.   The pharmaceutical composition of claim 68 or 69 in which the protein, peptide, or derivative is glycosylated.

80.   The pharmaceutical composition of claim 68 or 69 in which the protein, peptide, or derivative is nonglycosylated.

81.   A pharmaceutical composition comprising an effective amount of an antibody that recognizes BDNF protein or a peptide fragment or derivative thereof.

82.   A pharmaceutical composition comprising an effective amount of a combination of substantially pure BDNF protein or peptide fragment or derivative thereof, and a second agent.

83. A pharmaceutical composition of claim 82 such that said combination exhibits synergism in a functional activity of BDNF.

5

84. The pharmaceutical composition of claim 82 in which the second agent is nerve growth factor.

85. The pharmaceutical composition of claim 82 in which the second agent is another member of the BDNF/NGF
10 family.

86. A pharmaceutical composition comrising an effective amount of a protein or a peptide or derivative thereof encoded by the recombinant DNA molecule of claim 54,
15 55, 56, 57, 58, or 59.

87. A method for diagnosing a disease or disorder of the nervous system comprising
20   (a) contacting a tissue with a detectably labeled nucleic acid molecule which contains at least ten nucleotides substantially as depicted in Figure 5, under conditions which will allow hybridization to occur; and
25   (b) detecting any hybridization which has occurred.

88. A method for diagnosing a disease or disorder
30 of the nervous system comprising
       (a) contacting RNA collected from a tissue with a detectably labeled nucleic acid molecule which contains at least 10

35

nucleotides substantially as depicted in
Figure 1, under conditions which will
allow hybridization to occur; and

(b) detecting any hybridization which has
occurred.

89.   A method for diagnosing a disease or disorder
of the nervous system comprising

(a)   contacting cDNA produced from RNA
collected from a tissue with a detectably
labeled nucleic acid molecule which
contains at least 10 nucleotides
substantially as depicted in Figure 1,
under conditions which will allow
hybridization to occur; and

(b)   detecting any hybridization which has
occurred.

90.   The method according to any of claims 87, 88,
or 89 in which the disease or disorder of the nervous system
is a selected from the group consisting of a tumor, a
degenerative disease, a retinal disease, and a sensory neuron
disease.

91.   The method according to claim 90 in which the
tumor is neuroblastoma.

92.   A method for diagnosing a disease or disorder
of the nervous system comprising

(a)   exposing a tissue to a detectably
labeled antibody molecule which is
capable of binding to BDNF protein or a

peptide fragment or derivative thereof
under conditions which will allow binding
to occur; and

5   (b)   detecting any binding which has
occurred.

93.   The method according to claim 92 which is
performed in vivo.

10
94.   The method according to claim 92 performed in
vitro.

95.   The method according to claim 92 in which the
disease or disorder of the nervous system is selected from
15 the group consisting of a tumor, a degenerative disease, a
retinal disease, and a sensory neuron disease.

96.   The method according to claim 95 in which the
tumor is a neuroblastoma.
20

97.   A method of treating a disease or disorder of
the nervous system comprising administering an effective
amount of BDNF protein, or a functionally active peptide
25 fragment or derivative thereof, to a patient.

98.   The method according to claim 97 in which the
disease or disorder of the nervous system is a degenerative
disease.

30
99.   The method according to claim 98 in which the
degenerative disease is retinal disease.

35

100.   The method according to claim 97 in which the disease or disorder of the nervous system comprises damage to the nervous system.

5

101.   The method according to claim 100 in which damage is caused by events selected from the group consisting of trauma, surgery, infarction, infection, and malignancy.

102.   The method according to claim 97 in which the

10   disease or disorder is caused by exposure to a toxic agent.

103.   The method according to claim 97 in which the disease or disorder is caused by nutritional deficiency.

15

104.   The method according to claim 94 in which the disorder comprises a congenital disorder of the retina.

105.   A method of treating a disease or disorder of the nervous system comprising administering an effective

20   amount of a combination of BDNF protein, or a functionally active peptide fragment or derivative thereof, and a second agent.

106.   The method according to claim 105 in which the

25   combination which combination exhibits synergism in a functional activity of BDNF to a patient.

107.   The method of claim 105 in which the second

30   agent is nerve growth factor.

108.   The method of claim 105 in which the second agent is another member of the BDNF/NGF family.

35

109. The method according to claim 107 in which the disease or disorder is a degenerative disease.

110. The method according to claim 109 in which the degenerative disease is selected from the group consisting of Alzheimer's disease, Huntington's chorea, retinal disease and Parkinson's disease.

111. The method according to claim 105 in which the disease or disorder comprises damage to the nervous system.

112. The method according to claim 105 in which the disorder is damage caused by an event selected from the group consisting of trauma, surgery, infarction, infection, malignancy, and toxic agents.

113. The method according to claim 105 in which the disease or disorder comprises a disease or disorder of sensory neurons.

114. The method according to claim 105 in which the disease or disorder comprises a congenital disorder of the retina.

115. A method for isolating a gene which is a member of the brain derived neurotrophic factor/nerve growth factor gene family, but which encodes neither nerve growth factor nor brain derived neurotrophic factor, comprising:

    (a) selecting, from among a diversity of nucleic acid sequences, those sequences that are homologous to both BDNF and NGF;

(b)   identifying, from among the nucleic acid
sequences selected in (a), those sequences which
contain sequences of about at least 6 contiguous
nucleotides that are not homologous to NGF and
BDNF; and

(c)   isolating the sequences identified in (b).

116.   The method according to claim 115 in which the
method of step (a) comprises using the polymerase chain
reaction technique, in which a pair of oligonucleotide
primers are used which are capable of hybridizing to
homologous regions of BDNF and NGF DNAs, such that the sense
strand oligonucleotide primer is capable of hybridizing to a
first homologous DNA sequence of BDNF and NGF, and the
antisense strand oligonucleotide primer is capable of
hybridizing to a second homologous DNA sequence of BDNF and
NGF.

117.   The method according to claim 116 in which the
sense strand oligonucleotide primer comprises a nucleic acid
sequence substantially as depicted in Figure 1, which is
selected from the group consisting of from about residue 587
to residue 616, from about residue 713 to residue 739, from
about residue 764 to residue 781, from about residue 863 to
residue 898, and sequences thereof; and the antisense strand
oligonucleotide primer comprises a nucleic acid sequence
substantially as depicted in Figure 1, which is (a) selected
from the group consisting offrom about residue 587 to about
residue 616, from about residue 713 to about 739, from about
residue 764 to about residue 781, from about residue 863 to
about residue 898, and (b) is different from the sense strand
primer.

118.   A method for isolating a gene which is a
member of the brain derived neurotrophic factor/nerve growth
factor gene family, but which encodes neither nerve growth
factor nor brain derived neurotrophic factor, comprising:

> (a)   selecting, from among a diversity of nucleic
> acid sequences, those sequences that are homologous
> to either BDNF or NGF and a gene encoding the amino
> acid sequence substantially as depicted in Figure
> 14, denoted as M3/4.
>
> (b)   identifying, from among the nucleic acid
> sequences selected in (a), those sequences which
> contain sequences of about at least 6 contiguous
> nucleotides that are not homologous to NGF and
> BDNF; and
>
> (c)   isolating the sequences identified in (b).

119.   The method according to claim 118 in which the
method of step (a) comprises using the polymerase chain
reaction technique, in which a pair of oligonucleotide
primers are used which are capable of hybridizing to
homologous regions of either BDNF or NGF and a gene encoding
the amino acid sequence substantially as depicted in Figure
14, denoted as M3/4, such that the sense strand
oligonucleotide primer is capable of hybridizing to a first
homologous DNA sequence of BDNF or NGF or the gene encoding
the amino acid sequence denoted as M3/4, or, alternatively,
Neurotrophin-3, or NT-3, and the antisense strand
oligonucleotide primer is capable of hybridizing to a second
homologous DNA sequence of BDNF and NGF or the gene encoding
M3/4.

120.   A method for increasing the levels of BDNF
expressed in nervous system cells comprising exposing the
nervous system cells to kainic acid or a related compound.

121.   A method for increasing the levels of BDNF expressed in nervous system cells comprising exposing the nervous system cells to a non-NMDA receptor agonist.

5

122.   A method for increasing the levels of NGF expressed in nervous system cells comprising exposing the nervous system cells to kainic acid or a related compound.

123.   A method for increasing the levels of NGF

10   expressed in nervous system cells comprising exposing the nervous system cells to a non-NMDA receptor agonist.

124.   A method of promoting the survival of dopaminergic neurons comprising exposing the neurons to

15   effective levels of BDNF.

125.   A method of promoting the survival of cholinergic neurons comprising exposing the neurons to effective levels of BDNF.

20

126.   The method according to claim 125 in which the cholinergic neurons are basal forebrain cholinergic neurons.

25

127.   The method according to claim 125 in which the cholinergic neurons are septal cholinergic neurons.

128.   A method of suppressing the proliferation of astroglial cells comprising exposing the astroglial

30   cells to an effective amount of BDNF.

35

129. A method of inhibiting the uptake of gamma aminobutyric acid in neurons comprising exposing the neurons to an effective amount of BDNF.

5

130. A method of upregulating the expression of NGF receptor on a cell surface comprising exposing the cell to an effective amount of BDNF.

10

131. A method of preparing active BDNF from a less active precursor molecule comprising exposing the precursor molecule to an effective amount of endoproteinase Arg-C.

15

132. The method according to claim 131 in which an effective amount of endoproteinase Arg-C is endoproteinase Arg-C at a concentration of 0.1 unit per microliter.

20

133. An active form of BDNF produced by the method of claim 131.

134. An active form of BDNF produced by the method of claim 132.

25

135. The method according to claim 98 in which the degenerative disease is Parkinson's disease.

136. The method according to claim 98 in which
30 the degenerative disease is Alzheimer's disease.

137. The method according to claim 105 in which the disorder is damage caused by a toxic agent.

35

138. The method according to claim 98 in which the degenerative disease is a "Parkinson-Plus" syndrome.

139. The method according to claim 138 in which the "Parkinson-Plus" syndrome is selected from the group consisting of Progressive Spranuclear Palsey (Steele-Richardson-Olszewski Syndrome), Olivopontocerebellar Atrophy, Shy-Drager Syndrome (Multiple Systems Atrophy), and Guamanian Parkinsonism-Dementia complex.

140. The method according to claim 135 in which symptoms of Parkinson's disease are caused by a toxic agent.

141. The method according to claim 99 in which retinal disease is caused by damage to the optic nerve.

142. A method for increasing the levels of BDNF expressed in nervous system cells comprising exposing the nervous system cells to carbachol.

143. A method for increasing the levels of BDNF expressed in nervous system cells comprising exposing the nervous system cells to histamine.

144. A method for increasing the levels of BDNF expressed in nervous system cells comprising exposing the nervous system cells to bradykinin.

145. A method for increasing the levels of NGF expressed in nervous system cells comprising exposing the nervous system cells to carbachol.

146. A method for increasing the levels of NGF expressed in nervous system cells comprising exposing the nervous system cells to histamine.

5

147. A method for increasing the levels of NGF expressed in nervous system cells comprising exposing the nervous system cells to bradykinin.

10

148. The method according to claim 98 in which the degenerative disease is Huntington's chorea.

15

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1/52

FIG. 1

```
                                                            AAACCGGG        8

CACCAAAGATTCCCCCCTACCCCTTCTTTTTGACCAAAGGGAACGTGAAAAAATAATAGAGTCTGGGGATTTCGGGGCC      84

GAAGTCTTCCCCAGAGCAGCTGCCTTGATGTTTACTTTGACAAGTAGTGACTGAAAAGTTCCACCAGGTGAGAAGAGTG     166
1                                     10                                    20
Met Thr Ile Leu Phe Leu Thr Met Val Ile Ser Tyr Phe Gly Cys Met Lys Ala Ala Pro
ATG ACC ATC CTT TTC CTT ACT ATG GTT ATT TCA TAC TTC GGT TGC ATG AAG GCT GCC CCC     226
                                      30                                    40
Met Lys Glu Ala Asn Val Arg Gly Gln Gly Ser Leu Ala Tyr Pro Gly Val Arg Thr His
ATG AAA GAA GCC AAC GTC CGA GGA CAA GGC AGC TTG GCC TAC CCA GGT GTG CGG ACC CAT     286
                                      50                                    60
Gly Thr Leu Glu Ser Val Asn Gly Pro Lys Ala Gly Ser Arg Gly Leu Thr Ser Ser Ser
GGG ACT CTG GAG AGC GTG AAT GGG CCC AAG GCA GGT TCA AGA GGC CTG ACA TCG TCG TCA     346
                                      70                                    80
Ser Ser Ser Leu Ala Asp Thr Phe Glu His Val Ile Glu Glu Leu Leu Asp Glu Asp Gln
TCG TCG TCG TTG GCG GAC ACT TTT GAA CAC GTG ATC GAG GAG CTG TTG GAC GAG GAC CAG     406
                                      90                                    90
Lys Val Arg Pro Asn Glu Glu Asn Asn Lys Asp Ala Asp Met Tyr Thr Ser Arg Val Met
AAA GTT CGG CCC AAT GAG GAA AAC AAT AAG GAC GCG GAC ATG TAT ACG TCC CGA GTC ATG    ·466
                                      110                                   120
Leu Ser Ser Gln Val Pro Leu Glu Pro Pro Leu Leu Phe Leu Leu Glu Glu Tyr Lys Asn
CTC AGC AGT CAA GTG CCT TTG GAG CCT CCT CTT CTC TTT CTG CTG GAG GAA TAC AAA AAT     526
                                      130                                   140
Tyr Leu Asp Ala Ala Asn Met Ser Met Arg Val Arg Arg His Ser Asp Pro Ala Arg Arg
TAC CTG GAT GCT GCA AAC ATG TCC ATG AGG GTC CGG CGC CAC TCG GAC CCC GCC CGC CGC     586
                                      150                                   160
Gly Glu Leu Ser Val Cys Asp Ser Ile Ser Glu Trp Val Thr Ala Ala Asp Lys Lys Thr
GGG GAG CTG AGC GTG TGC GAC AGC ATT AGC GAG TGG GTG ACG GCG GCG GAT AAA AAG ACG     646
                                      170                                   180
Ala Val Asp Met Ser Gly Gly Thr Val Thr Val Leu Glu Lys Val Pro Val Ser Lys Gly
GCA GTG GAC ATG TCG GGT GGC ACG GTC ACG GTC CTC GAA AAA GTC CCC GTC TCG AAA GGC     706
                                      190                                   200
Gln Leu Lys Gln Tyr Phe Tyr Glu Thr Lys Cys Asn Pro Met Gly Tyr Thr Lys Glu Gly
CAA CTG AAG CAG TAC TTC TAC GAG ACC AAG TGC AAT CCT ATG GGG TAC ACA AAG GAG GGC     766
                                      210                                   220
```
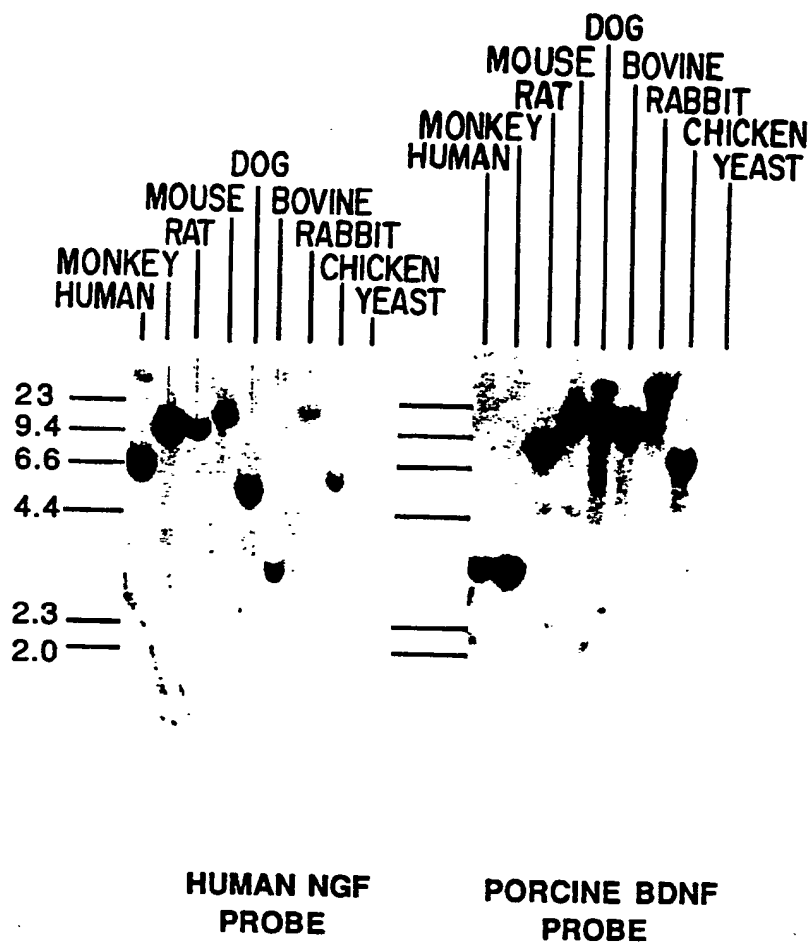
FIG. 1 (cont.)

```
Cys Arg Gly Ile Asp Lys Arg His Trp Asn Ser Gln Cys Arg Thr Thr Gln Ser Tyr Val
TGC AGG GGC ATA GAC AAG AGG CAC TGG AAC TCC CAG TGC CGA ACT ACC CAG TCG TAT GTG    826
                                 230                                           240
Arg Ala Leu Thr Met Asp Ser Lys Lys Arg Ile Gly Trp Arg Phe Ile Arg Ile Asp Thr
CGG GCC CTC ACC ATG GAT AGC AAA AAA CGA ATT GGC TGG CGG TTC ATA AGG ATA GAC ACT    886
                                 250
Ser Cys Val Cys Thr Leu Thr Ile Lys Arg Gly Arg End
TCC TGT GTA TGT ACT TTG ACC ATT AAG AGG GGA AGA TAG TGGCTTTATGTTGTATAGATTATATTG    952

AGACAAAAATTATCTATTTGTATATATACATAACAGGGTAAATTATTCAGTTAAGAAAAAAAATAATTTTATGAACTGC  1031

ATGTATAAATGAAGTTTATACAGTACAGTGGTTCTACAATCTATTTATTGGACATTTCCATGACCAGAGGGAAACAGTC  1110

ATTTTTTGCGCACAACTTTAAAAAAAAAAGTCTGCATTACATTCCTCGATAATGTTGTGGTTTGTTGCCGTTGCT       1184
```

FIG. 2

```
HSDPARRGELSVCDSISEWVTAADKKTAVDMSGGTVTVLEKVPVSKGQLKQYFYETKCNP    pig BDNF
SSSHPIFHRGEFSVCDSVSVWV..GDKTTATDIKGKEVMVLGEVNINNSVFKQYFFETKCRD    human NGF
SSSHPVFHRGEFSVCDSISVWV..GDKTTATDIKGKEVMVLGEVNINNSVFKQYFFETKCRD    bovine NGF
SSTHPVFHMGEFSVCDSVSVWV..ADKTTATDIKGKEVTVLAEVNVNNNVFKQYFFETKCRD    g. pig NGF
SSTHPVFHMGEFSVCDSVSVWV..GDKTTATDIKGKEVTVLAEVNINNSVFRQYFFETKCRA    mouse NGF
TAHPVLHRGEFSVCDSVSMWV..GDKTTATDIKGKEVTVLGEVNINNNVFKQYFFETKCRD    chick NGF
EDHPVHNLGEHSVCDSVSAWV...TKTTATDIKGNTVTVMENVNLDNKVYKQYFFETKCKN    snake NGF


MGYTKEGCRGIDKRHWNSQCRTTQSYVRALTMDSKKRIGWRFIRIDTSCVCILTIKRGR     pig BDNF
PNPVDSGCRGIDSKHWNSYCTTTHTFVKALTMDGKQ.AAVRFIRIDTACVCVLSRKAVRRA   human NGF
PNPVDSGCRGIDAKHWNSYCTTTHTFVKALTMDGKQ.AAVRFIRIDTACVCVLSRKTGQRA   bovine NGF
PSPVESGCRGIDSKHWNSYCTTTHTFVKALTTDNKQ.AAVRFIRIDTACVCVLNRKAARRG   g. pig NGF
SNPVESGCRGIDSKHWNSYCTTTHTFVKALTTDEKQ.AAVRFIRIDTACVCVLSRKATRRG   mouse NGF
PRPVSSGCRGIDAKHWNSYCTTTHTFVKALTMCGKQ.AAVRFIRIDTACVCVLSRKSGRP    chick NGF
PNPEPSGCRGIDSSHWNSYCTETDTFIKALTMEGNQ.ASVRFIRIETACVCVITKKKGN     snake NGF
```

FIG. 3

FIG. 4

FIG. 5

FIG.5 (cont.)
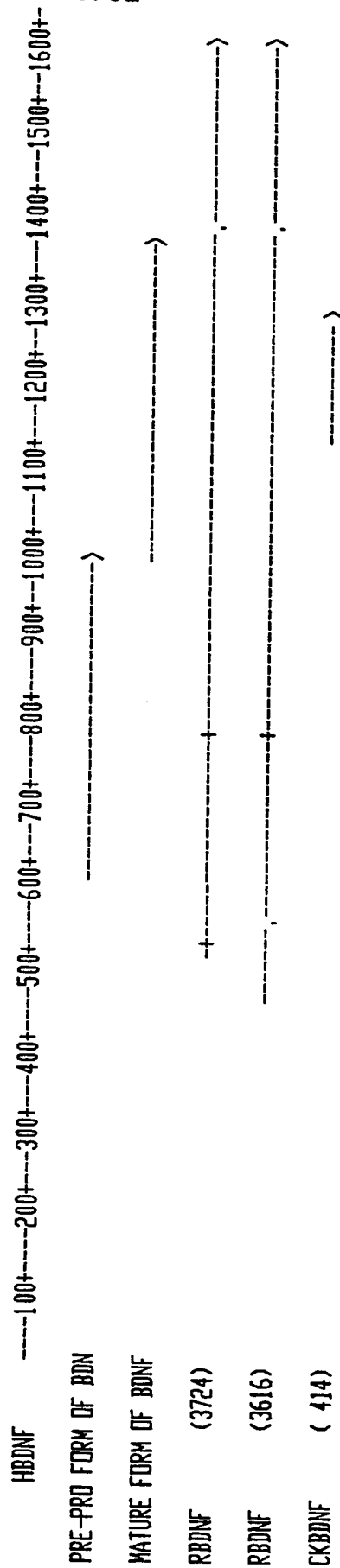
```
           10        20        30        40        50        60        70        80        90       100       110       120

HUMAN  AAGCTTGATATGCGAATTCCGGAATTCCGTTCCCAACTGCTGTTTATTGTGCTATTCATGCCTAGACATACATAGCTAGAAAGGCCCATCAGACCCTCAGCCACTGCTGTTCCTGTCACACATTC
       TTCGAACTATAGCTTAAGGCCTTAAGGCAAGGGGTTGACGACAAAATAACACGATAAGTACGATCTCTGAGTGTATCGGATCTTTCCGGGTAGTCTGGGAGTCCGGTGACGACAAGGACAGTGTGTAAG

          130       140       150       160       170       180       190       200       210       220       230       240       250

HUMAN  CTGCAAAGGACCATGTTGCTAACTTGAAAAAAATTACTATTAATTAATTACACTTCGCAGTTGTTGTTGCTTAGTAGCATTTATGATTTGTGTTCTCTGTGACAGCATGAGCAGAGATCATTAAAAATTAAACTTA
       GACGTTCCTGGTACAACGATTGAACTTTTTTAATGTGAAACTTAAATTAAATGATAATTAATCATTGTAAAACTAAAACACAAAGAGCACTGTGTACTCGTCTCAGTAATTTTTAATTTGAAT

          260       270       280       290       300       310       320       330       340       350       360       370       380

HUMAN  CAAAGCTGCTAAAGTGGGAAGAAGGAGAAGAGAACTTGAAGCCACCAATTTTTGCACTTGCTTAGAAGCCATCTAATCTCAGGTTATATGCTAGATCTTGGGGGCAAACACTGCGATCTTGCTCTGGTTTATATTAAA
       GTTTCGACGATTTCACCCTTCTTCCTCTCTGAACTTCGGTGTTAAAAACGTGAACGAATCTTCGGTAGATTAGAGTCCAATATACGATCTAGAAACCCGTTTGTGACGTACAGAGACCAAATATAATTT

                                                                    ∟HBDNF

          390       400       410       420       430       440       450       460       470       480       490       500       510

HUMAN  CCACATACAGCACACTACTGACACTGATTTGTGTCTGGTCGGTCAGTGTCTGCGAGCTGGAGTTTATCATCCAAGACATAAAAAAAACTTGACCCTGCAGAATGGCTGGATTTTCACCACCAGGAATTACAATCAGATGGGCCACATGGCATCCGG
       GGTGTATGTCGTGTGATGACTGTGACTAAACAGACCAGCTGTCGACCTCAAATAGTGGTTCTGTATTTTTTGGAACTGGGACGTCTTACCGGACCTTAATGTGTTAGTCTACCCGGTGTACCGTAGGGC

                                                                                                                                   C

                                                                                         ..CA..GGG...G.G..AAAATA.T.GA.T.TG..G..     .-..A..T....>
RAT   4
PIG  39                                                                                                                            --..-.--....G.A.T.T....-C>
```
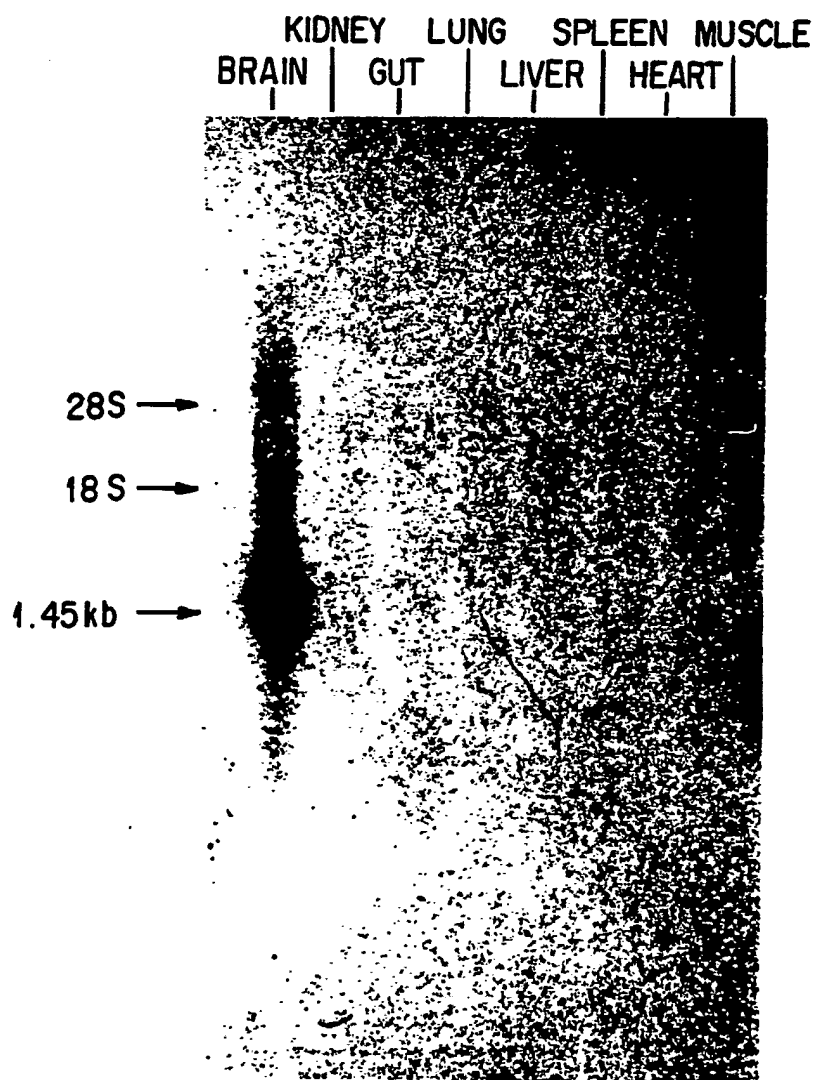
|——————HBDNF——————

HUMAN 520   530   540   550   560   570   580   590   600   610   620   630

TGAAAGAAAGCCTAACCAGTTTCTGTCTTGTTTCTGCTTTCTCCCTACAGTTCCACCAGGTGAGAAGAGTG ATG ACC ATC CTT TTC CTT ACT ATG GTT ATT TCA TAC TTT GGT
ACTTCTTTCGGGATTGGTCAAAAGACAGAACAAAGACGAAAGAGGATGTCAAGGTGTCCACTCTTCTCAC TAC TGG TAG AAG GAA TGA TAC CAA TAA AGT ATG AAA CCA
                                                                        Met Thr Ile Leu Phe Leu Thr Met Val Ile Ser Tyr Phe Gly)

                                                                        __α__α__α_PRE-PRO FORM OF BDNF__α__α__α__α_>

RAT 17 CCTTG..C..AG.-C.G.G.A...G.-C.GA.G.GG.-AG.A..T.A.C..C ....)
PIG 99 A..GCAGCT...T.G.--T....A..-.-GACAAG.AG.GA..-AAA.A..C ....)

FIG. 5 (cont.)

HUMAN

| 640 | 650 | 660 | 670 | 680 | 690 | 700 | 710 | 720 |

```
TGC ATG AAG GCT GCC CCC ATG AAA GAA GCA AAC ATC CGA GGA CAA GGT TTG GCC TAC CCA GGT GTG CGG ACC CAT GGG ACT CTG GAG AGC GTG
ACG TAC TTC CGA CGT GGG TAC TTT CTT CGT TTG TAG GCT CCT CGT TTG TAG GCT CCT CCA CCG AAC CGG ATG GGT CCA CAC GCC TGG GTA CCC TGA GAC CTC TCG CAC
Cys Met Lys Ala Ala Pro Met Lys Glu Ala Asn Ile Arg Gly Gln Gly Leu Ala Tyr Pro Gly Val Arg Thr His Gly Thr Leu Glu Ser Val
```
[HBDNF] PRE-PRO FORM OF BDNF

RAT 129 ....G .... G... .AC ....C AA. ..... .C ....

PIG 209 ....C ...... G... ....C A.. ....

HUMAN

| 730 | 740 | 750 | 760 | 770 | 780 | 790 | 800 | 810 | 820 |

```
AAT GGG CCC AAG GCA GGT TCA AGA GGC TTG ACA TCA TTG GCT GAC ACT TTC GAA CAC GTG ATA GAA GAG CTG TTG GAT GAG GAC CAG AAA GTT CGG
TTA CCC GGG TTC CGT CCA AGT TCT AGT TGT AAC CTT CGA AAG CTT AGT TGA AGC TTG CTG CAC TTC CTG GAC CTA CTC GTC TTT CAA GCC
Asn Gly Pro Lys Ala Gly Ser Arg Gly Leu Thr Ser Leu Ala Asp Thr Phe Glu His Val Ile Glu Glu Leu Leu Asp Glu Asp Gln Lys Val Arg
```
[HBDNF] PRE-PRO FORM OF BDNF

CGACGA
^

ATCGTCGTCATCGTC
^

RAT 225 ....G ..... .T C.. ..G ..C C... ...... .T .G ....C ....

PIG 305 .... C.. ..G .G ...... .G ...... .T ....C .G ....C ....

FIG. 5(cont.)

|HBDNF|

HUMAN

830   840   850   860   870   880   890   900   910

CCC AAT GAA GAA AAC AAT AAG GAC GAC TTG TAC ACG TCC AGG GTG ATG CTC AGT AGT CAA GTG CCT TTG GAG CCT CCT CTT CTC TTT CTG CTG
GGG TTA CTT CTT TTG TTA TTC CTG CGT CTG AAC ATG TCC AGG TCC AGG TCA TCA GAG TAC CAC GTT TCA GGA AAC CTC GGA GGA GAA GAC GAC
Pro Asn Glu Glu Asn Asn Lys Asp Asp Leu Tyr Thr Ser Arg Val Met Leu Ser Ser Gln Val Pro Leu Glu Pro Pro Leu Leu Phe Leu Leu

|PRE-PRO FORM OF BDNF|

RAT 327 ... ..C ... ... C... ... ... .G ... ... .C ... .T ... .C. ... ...
PIG 416 ... ... .G ... ... .G ... ... .G ... A.. .T A.. .C .C.A .C ... ... .G ... ...

FIG. 5 (cont.)

```
          920       930       940       950       960       970       980       990       1000      1010
HUMAN  GAG GAA TAC AAA AAT TAC CTA GAT GCT GCA AAC ATG TCC ATG AGG GTC CGG CGC CGA GGG GAG CTG AGC GTG TGT GAC
       CTC CTT ATG TTT TTA ATG GAT CTA CGA CGT TTG TAC AGG TAC TCC AGG TAC TCC CAG GCC GCG GCG GCT CCC CTC GAC TCG CAC ACA CTG
       Glu Glu Tyr Lys Asn Tyr Leu Asp Ala Ala Asn Met Ser Met Arg Val Arg Arg   His Ser Asp Pro Ala Arg Arg Gly Glu Leu Ser Val Cys Asp
       _a_a_a_a_a_PRE-PRO FORM OF BDNF_a_a_a_a_a_a HBDNF _b_b_b_b_b_b_b_MATURE FORM OF BDNF _b_b_b

RAT 423 ...  ...  ...  ...  ...  ...T ...  ...  ...C ...  .C ...  ...T ...  ...  .C ...
PIG 512 ...  ...  ...  .G ...  .G ...  ...  ...  ...  .G ...  ...  .C ...  ...  .C ...

          1020      1030      1040      1050      1060      1070      1080      1090      1100      1110
HUMAN  AGT ATT AGT GAG TGG GTA ACG GCG GCA GAC AAA AAG ACT GCA GTG GAC TCG GGC GGG ACG GTC ACA GTC CTT GAA AAG GTC CCT GTA TCA AAA
       TCA TAA TCA CTC ACC CAT TGC CGC CGT CTG TTT TTC TGA CGT CAC CTG TAC AGC CCG TAC AGC TGT CAG CCC TGC AGC CTT GAA GGA CAT AGT TTT
       Ser Ile Ser Glu Trp Val Thr Ala Ala Asp Lys Lys Thr Ala Val Asp Ser Gly Gly Thr Val Thr Val Leu Glu Lys Val Pro Val Ser Lys
       _b_b_b_b_b_b_b_b_b_b_MATURE FORM OF BDNF_b_b_b_b_b_b_b_b_b_b_b

RAT 519 ...  .C ...  ...  ...C ..A ...  ..T ...  ...  ...C ..T ...  ...  .G .G ..A ...  .G ...
PIG 608 ..C ...  .C ...  ...  .G ..T ...  .G .T ...  .T .C ...  ...  .C ...  ..A ...  .C .C ..G ...
```

FIG.5(cont.)

HUMAN

|_____HBDNF_____|
1120   1130   1140   1150   1160   1170   1180   1190   1200

GGC CAA CTG AAG CAA TTC TAC GAG ACC AAG TGC AAT CCC ATG GGT TAC ACA AAA GAA GGC TGC AGG GGC ATA GAC AAA AGG CAT TGG AAC TCC
CCG GTT GAC TTC GTT ATG ATG ACC TCG TTC ACG CTC TGG ATG ATG GGG TAC CCA TTA TCC ACG TGT CCG TAT CCG TCC CCG TAT CTG TCC TAT CTG AGG
Gly Gln Leu Lys Gln Tyr Phe Tyr Glu Thr Lys Cys Asn Pro Met Gly Tyr Thr Lys Glu Gly Cys Arg Gly Ile Asp Lys Arg His Trp Asn Ser
 b   b   b   b   b   b   b   b   b   b   b   MATURE FORM OF BDNF   b   b   b   b

RAT   615 ... ...T ... ...G ..G ... ...G
PIG   704 ... ... .G. ... ...T ... ... .G ... ..G ... ..C ... ...G
CHICKEN 1 ... ...T ...A ..A ... .T .A. .G ... ..G ...C ... ...A

HUMAN

|_____HBDNF_____|
1210   1220   1230   1240   1250   1260   1270   1280   1290   1300

CAG TGC CGA ACT ACC CAG TCG CGG GCC CTT ACC ATG GAT AGC AAA AAG AGA ATT GGC TGG CGA TTC ATA AGG ATA GAC ACT TCT TGT GTA
GTC ACG GCT GTC GTC AGC ATG TGG TAC CTA TTT TTC TCT AAA CCG GCT AAG TAT CTG TGA AGA ACA CAT
Gln Cys Arg Thr Thr Gln Ser Tyr Val Arg Ala Leu Thr Met Asp Ser Lys Lys Arg Ile Gly Trp Arg Phe Ile Arg Ile Asp Thr Ser Cys Val
 b   b   b   b   b   b   b   MATURE FORM OF BDNF   b   b   b   b   b   b   b   b   b   b

FIG. 5(cont.)

```
RAT     711  ..A ... ... ... ...A ... ..T ..T ... ... ...T ... ... ... ... ... ... ...G ... ... ... ... ...C ... ... ...
PIG     800  ... ... ... ... ... ... ..T ... ..T ... ...C ... ... ... ... ... ...G ... ...C ... ... ...
CHICKEN  91  ... ... ... ... ...T ... ... ... .A C... ... ... ... ... ...
```

|———————————————————————HBDNF|

```
             1310        1320        1330        1340        1350        1360        1370        1380        1390        1400        1410        1420

HUMAN    TGT ACA TTG ACC ATT AAA AGG GGA AGA TAGTGGATTTATGTTGTATAGATTAGATTATATTGAGACAAAAATTATCTATTGTATATACATAACAGGGTAAATTATTCAGTTAAGAAA
         ACA TGT AAC TGG TAA TTT TCC CCT TCT ATCACCTAAATACAACATATCTAATAATAACTCTGTTTTTAATAGATAAACATATATGTATTGTCCCATTTAATAAGTCAATTCTTT
         Cys Thr Leu Thr Ile Lys Arg Gly Arg
         |_b__ MATURE FORM OF BDNF _b__b____|

RAT      807  ... ... ...C ... ... ... ... ... ... ... ... ... ... ... ...
PIG      896  ... ..T ... ... ...G ... ... ...C ... ... ... ... ... ... ... ... ...A ‹
```

FIG. 5 (cont.)

```
                1430    1440    1450    1460    1470    1480    1490    1500    1510    1520    1530    1540    1550
                                                                        ⌐HBDNF⌐

HUMAN   AAAATAATTTATGAACTGCATGTATAAATGAAGTTTATACAGTACAGTGGTTCTACAACTCATTTATTATTGGACATGTCCATGACCAGAAGGGAAACAGTCATTTGCGCACAACTTAAAAAGTCTGCATT
        TTTTATTAAAAATACTTGACGTACACATATTTACTTCAAATATGTCAAATATGTCATGTCAAGAGATGTTAGATAAATAACCTGTACAGGTACTGGTCTTCCCTTTGTCAGTAAACGCGTGTTGAATTTTTCAGACGTAA

RAT 922 ..G...............................................................................A.........................A..................T.............T..........
                                                                                                              TTT     TTTAA
                                                                                                              ^       ^
PIG 1012 ..................................................................................T..........................._................AA.................


                1560    1570    1580    1590    1600    1610
                                              ⌐HBDNF⌐

HUMAN   ACATTCCTTGATAATGTTGTGGGTTTGTTGCCGTTGCCAAGAACTGAAAAACTGAAAAACGGAATTCCTGCAG
        TGTAAGGAACTATTACAACACCAAACAACGGCAACGGTTCTTGACTTTTGCCTTAAGGACGTC

RAT 1050 .........C...................................................T......AA..

PIG 1140 .........C.............................................
```

FIG. 5 (cont.)

Xbal
5'-ATAATCTAGATGACCATCCTTTTCCTT-3'
        MetThrIleLeuPheLeu

             IleLysArgGlyArgEND
3'-TAATTCTCCCCTTCTATCAGATCTAATA-3'
                              Xbal

1. PCR PORCINE GENOMIC DNA
2. CUT PRODUCT WITH Xbal
3. LIGATE 765 bp FRAGMENT INTO pCMV1

pCMV1-pBDNF (SENSE)
pCMV1-pBDNF (ANTI-SENSE)

CMV   Xbal   hGH TERM.   SV40 ori

pCMV1-pBDNF

FIG. 6

FIG. 7A

FIG. 7 B

FIG.8

18/52

FIG. 9

FIG. 10A

FIG. 10 B

FIG.11

FIG. 12A

FIG.12B

FIG. 15

FIG. 16A

FIG. 16B

FIG.17

FIG. 18

FOLD INCREASE BDNF mRNA

(KAINIC AC.) (μM)

FIG. 19 A

FIG. 19B

FIG. 20

FIG.21A

FIG.21B

FIG.21C

FIG.21D

FIG.21E

FIG.21F

FIG. 21 G
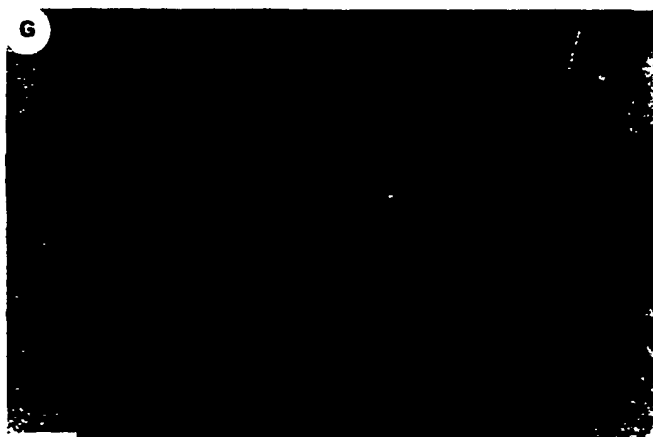
FIG. 21 H
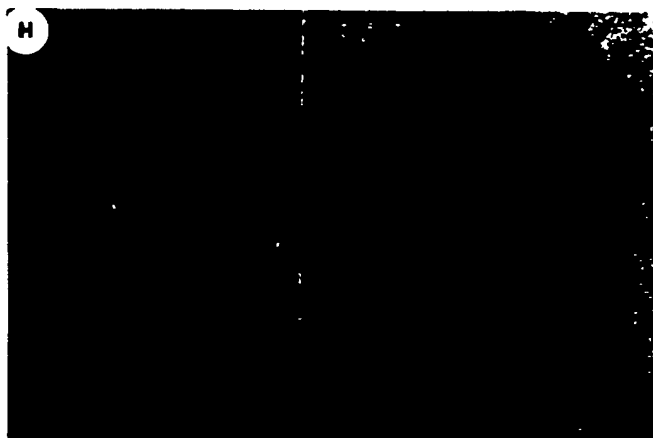
FIG. 21 I
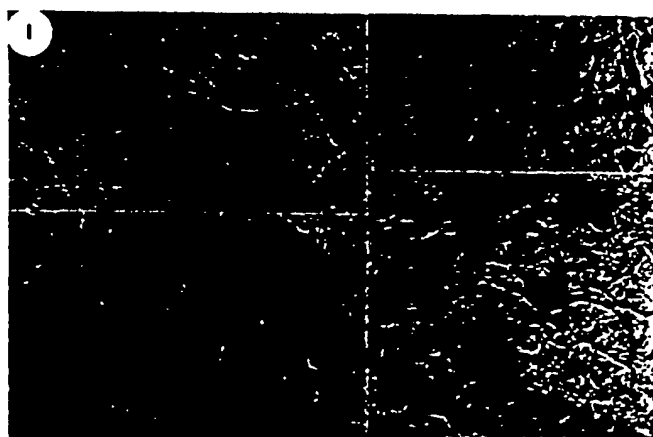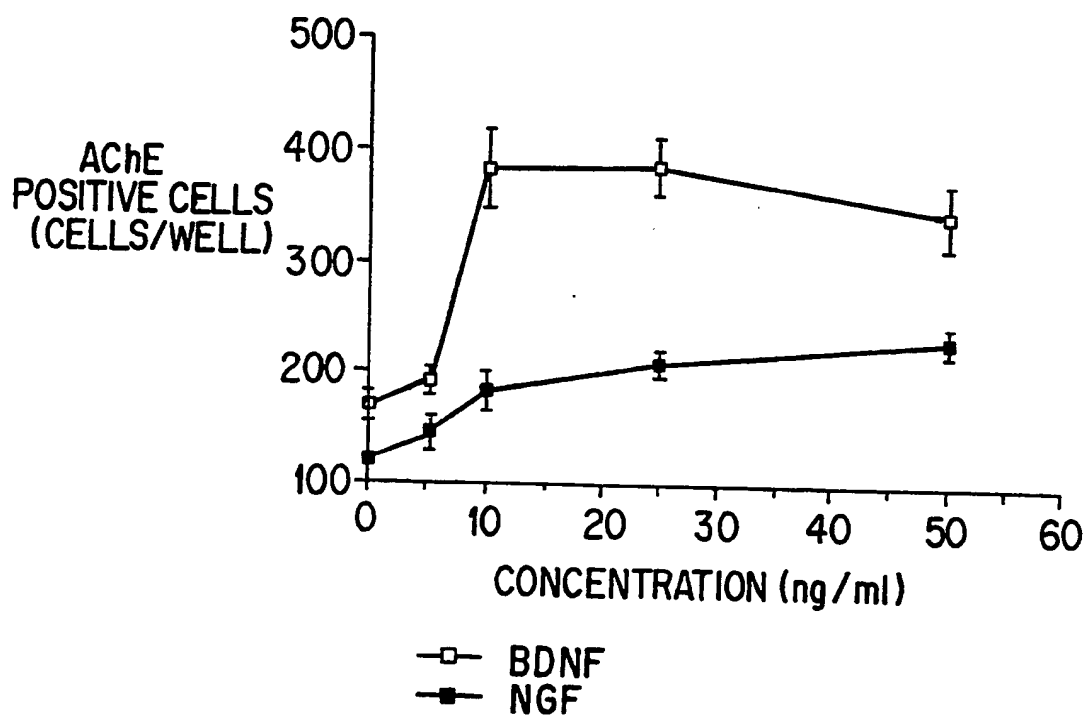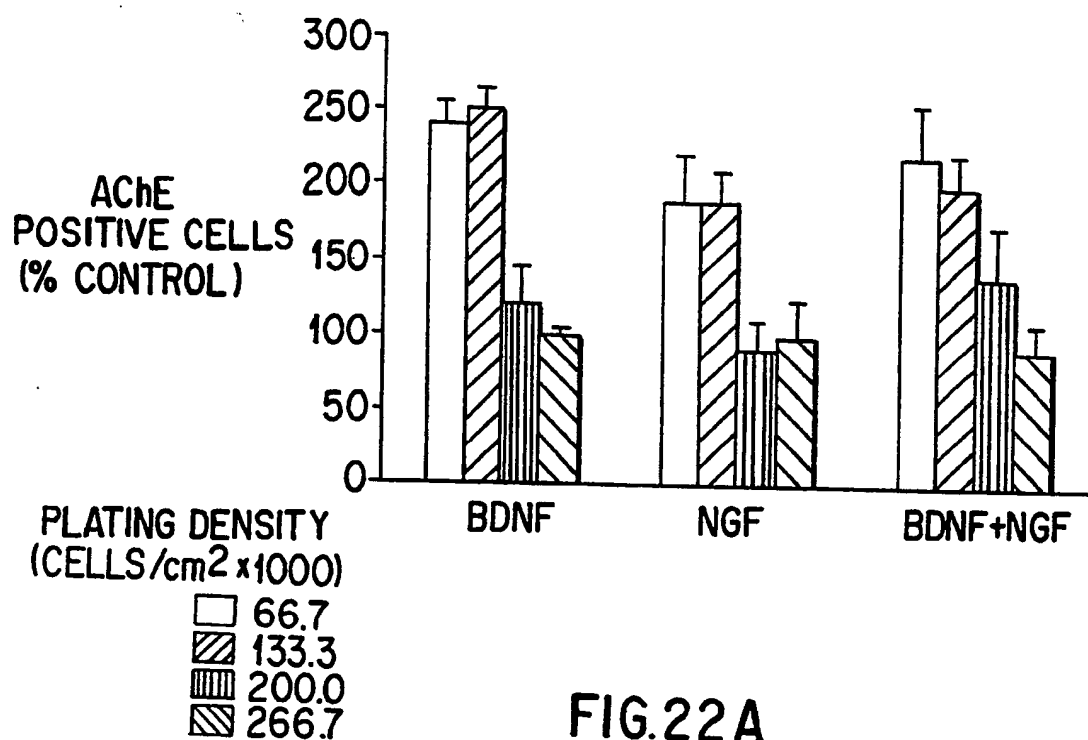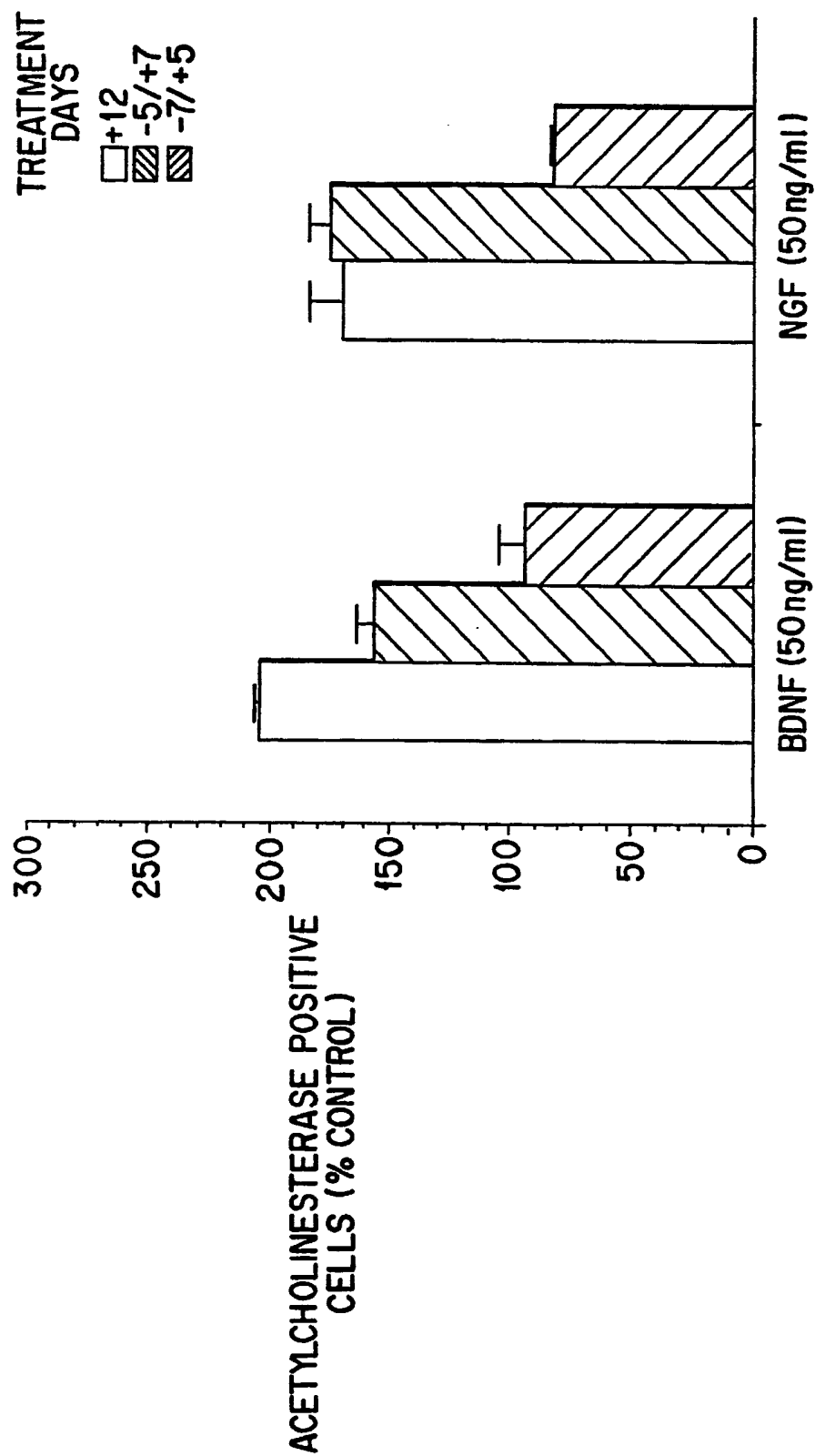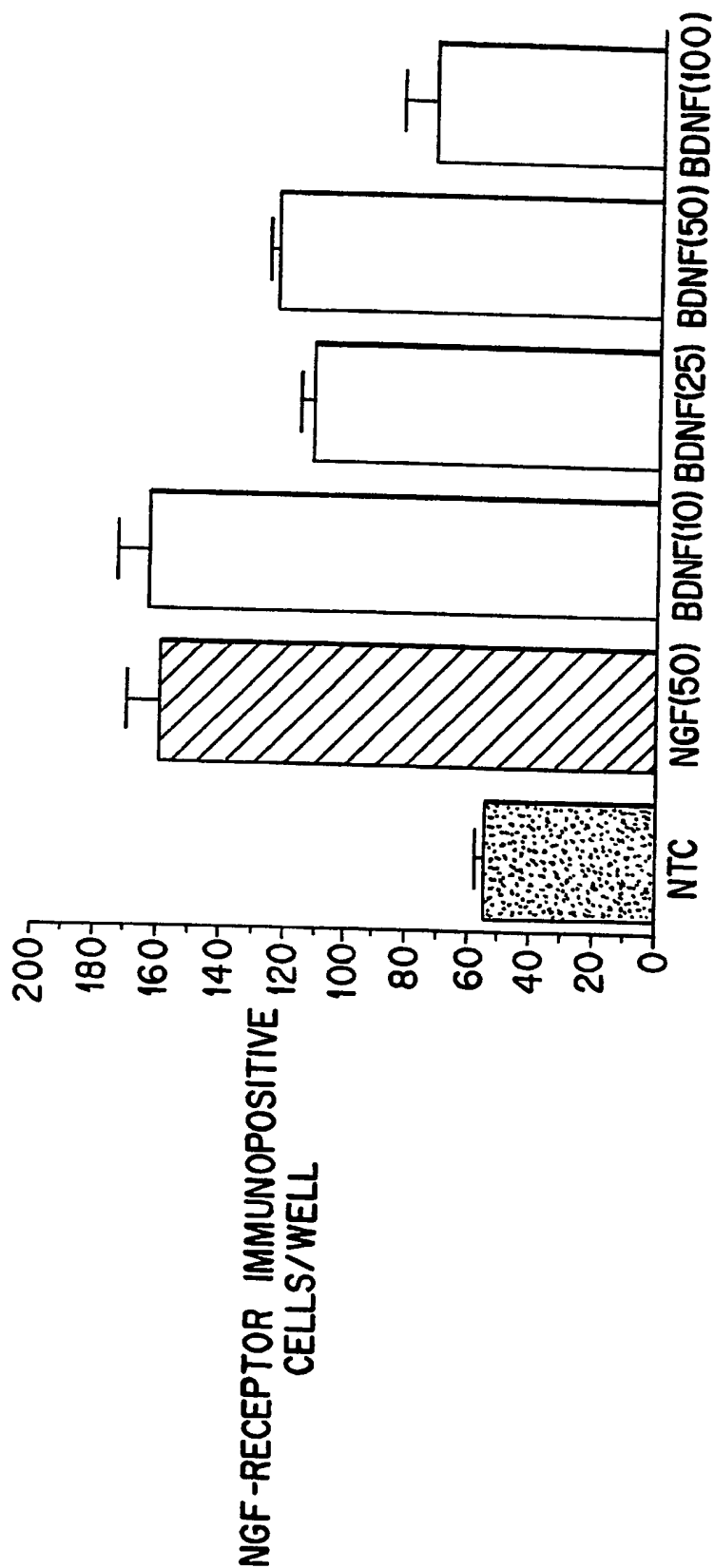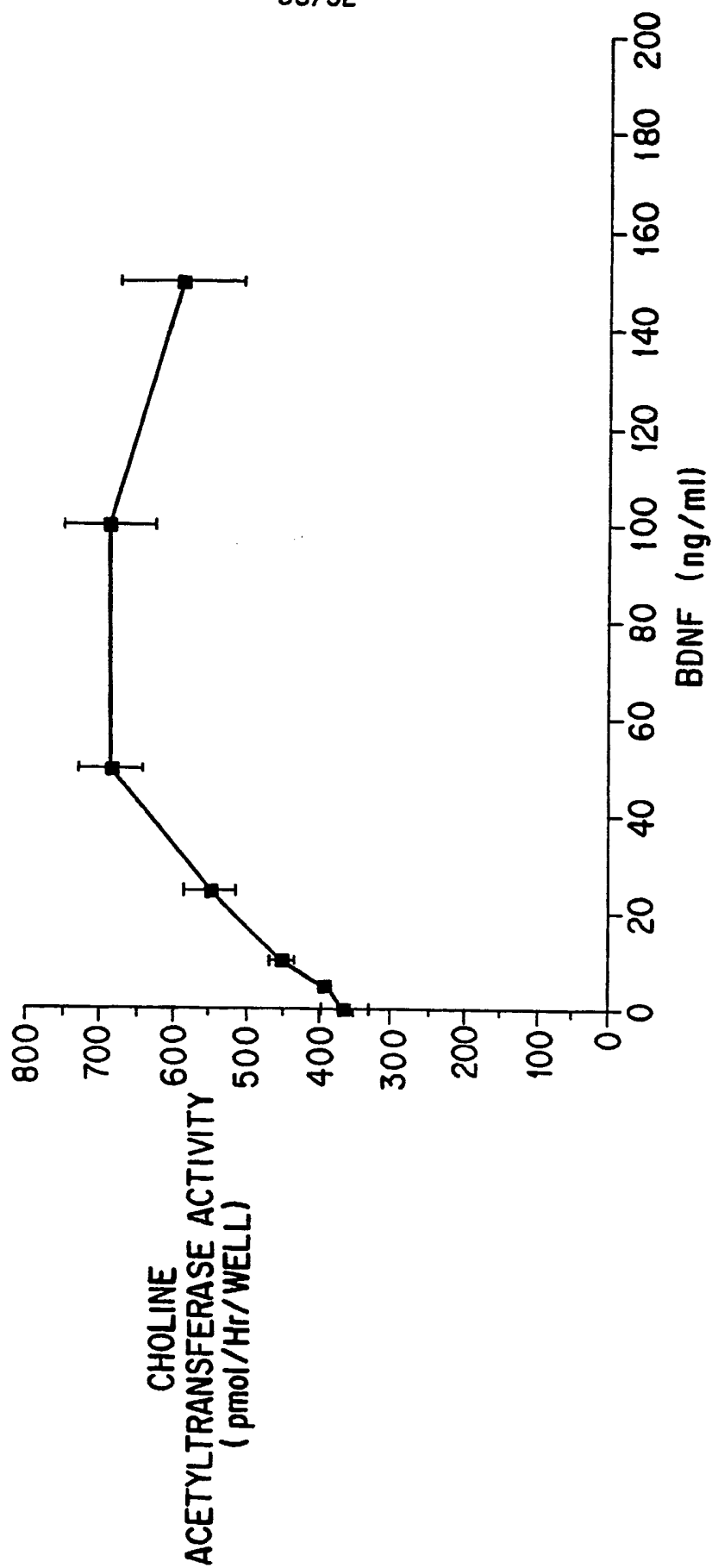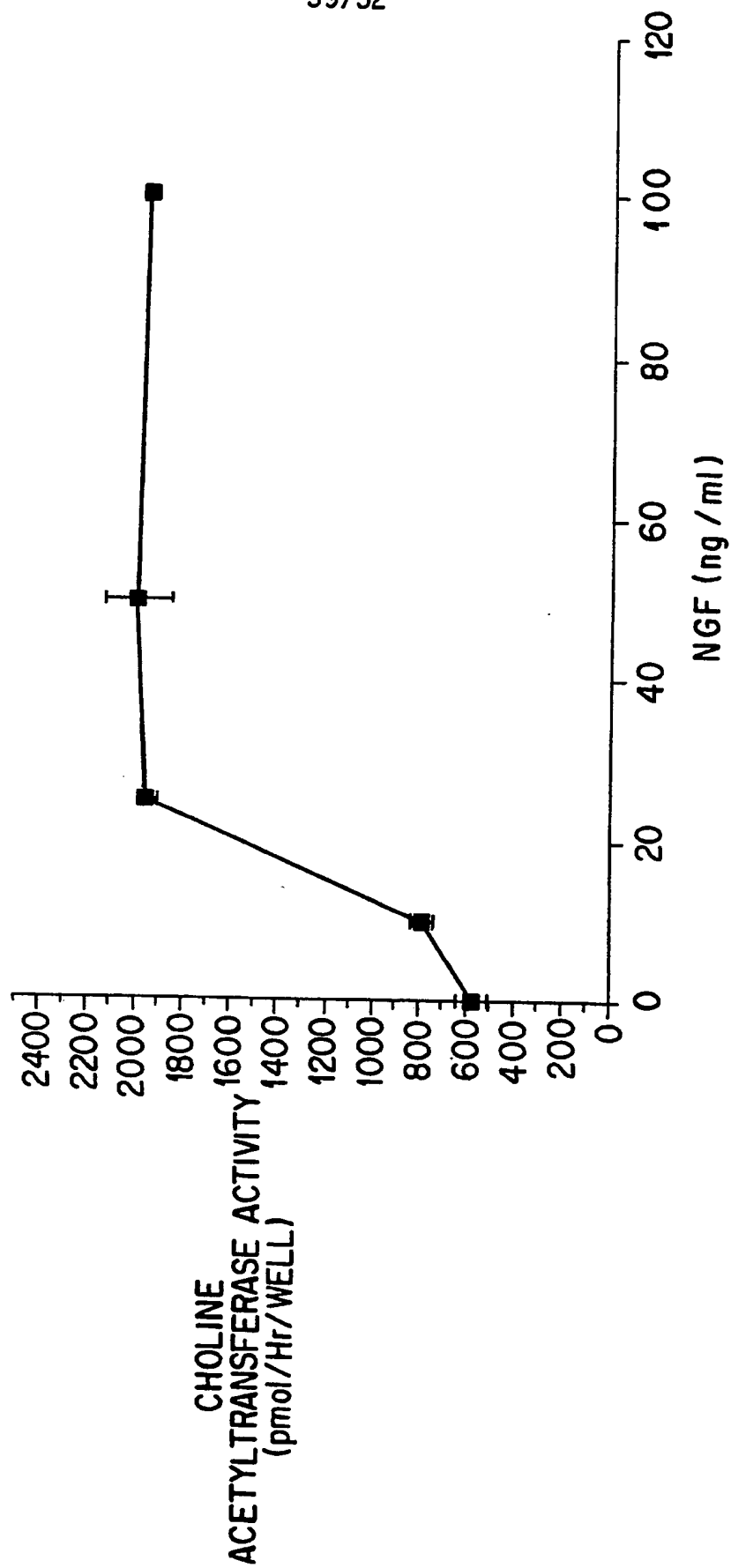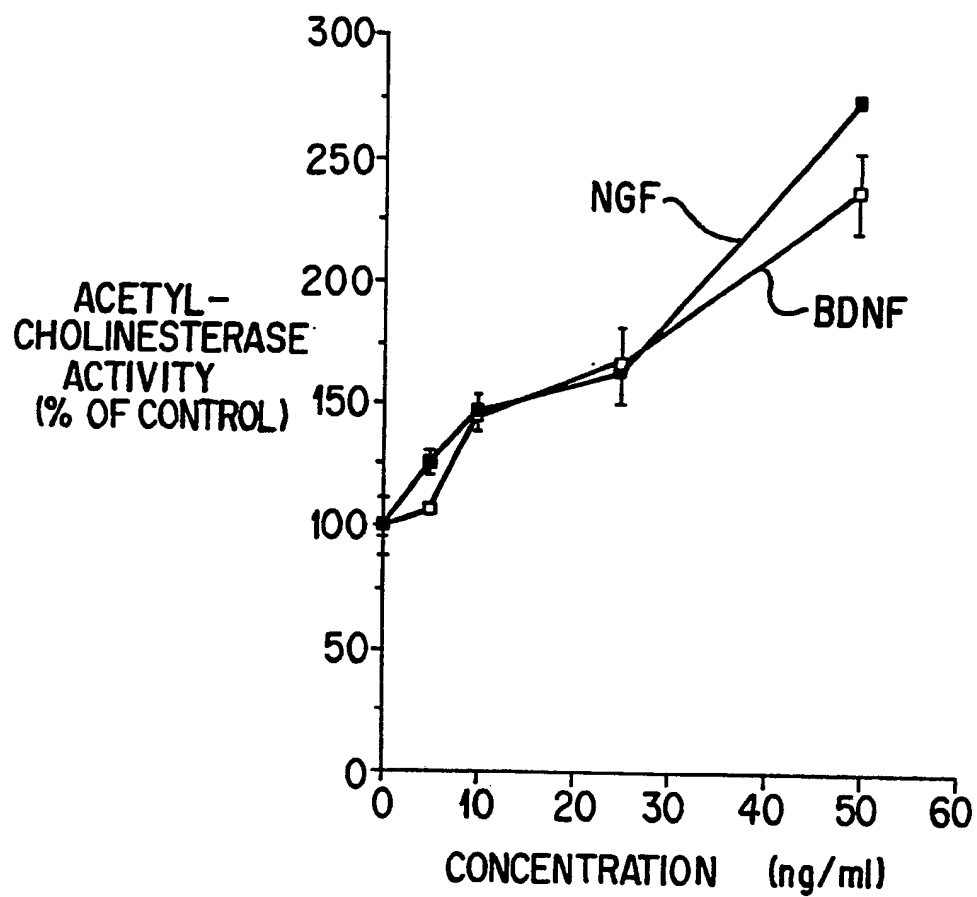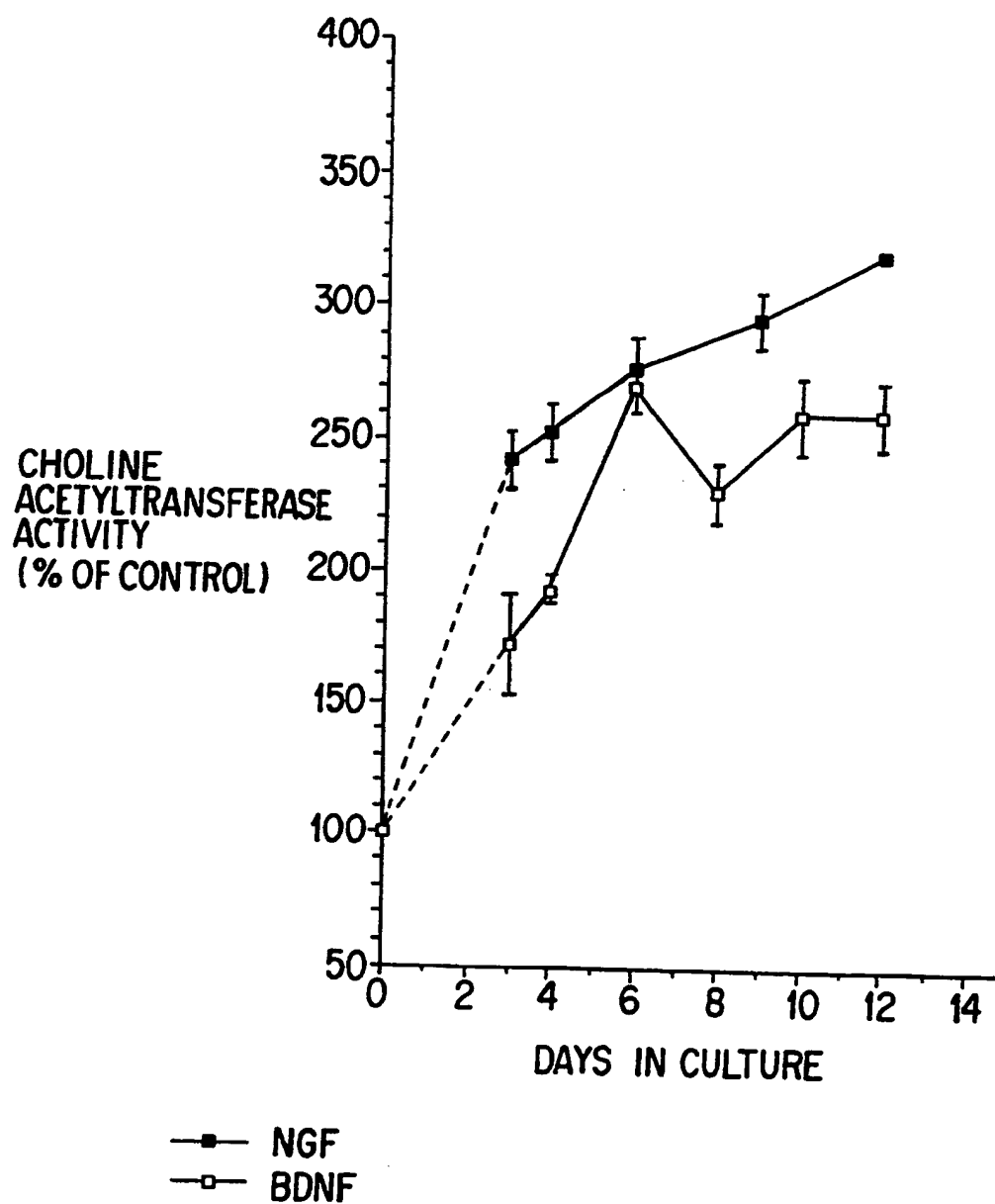
FIG.22A

FIG. 22B

FIG. 23

FIG. 24

FIG. 25A

CHOLINE
ACETYLTRANSFERASE ACTIVITY
(pmol/Hr/WELL)

BDNF (ng/ml)

FIG. 25B

FIG. 26

FIG. 27

FIG. 28

FIG. 29

FIG. 30

□ CONTROL
▨ NGF

TH POSITIVE NUMBER
(CELLS/DISH)

FIG. 34C

●—● UNTREATED BDNF
▲—▲ CLEAVED BDNF

NEUTRITE
OUTGROWTH
(ARBITRARY
UNITS)

CHO BDNF (μl)
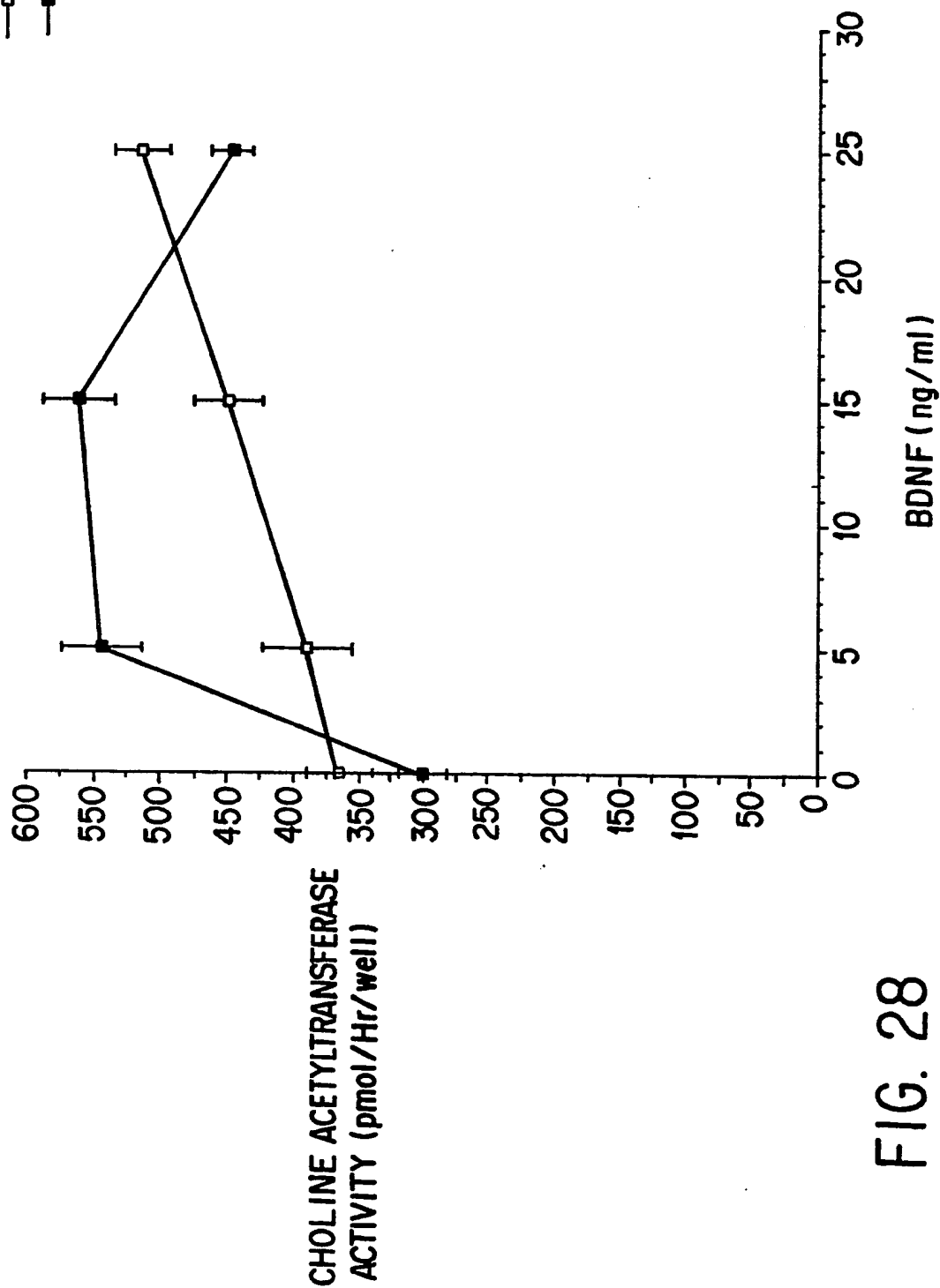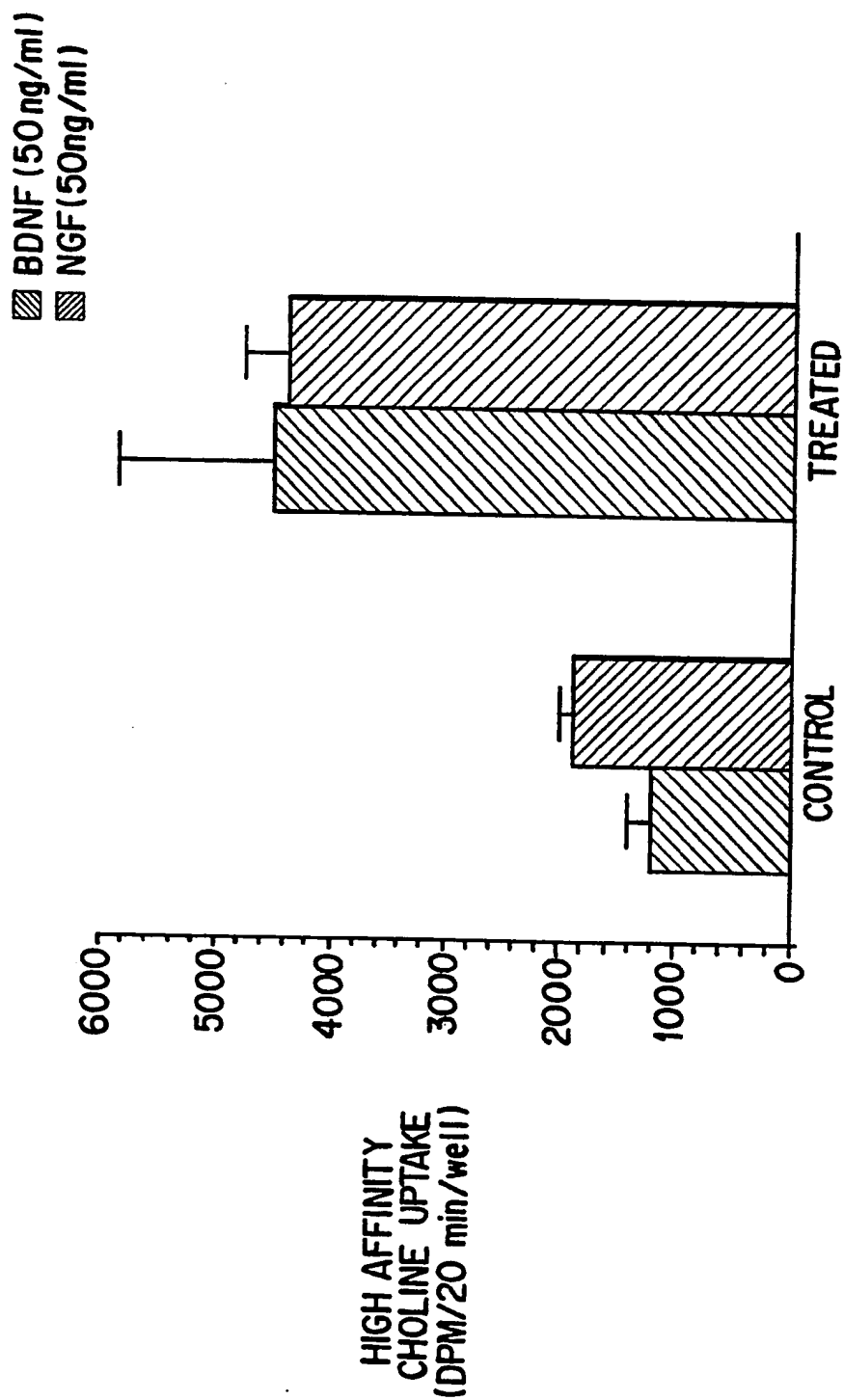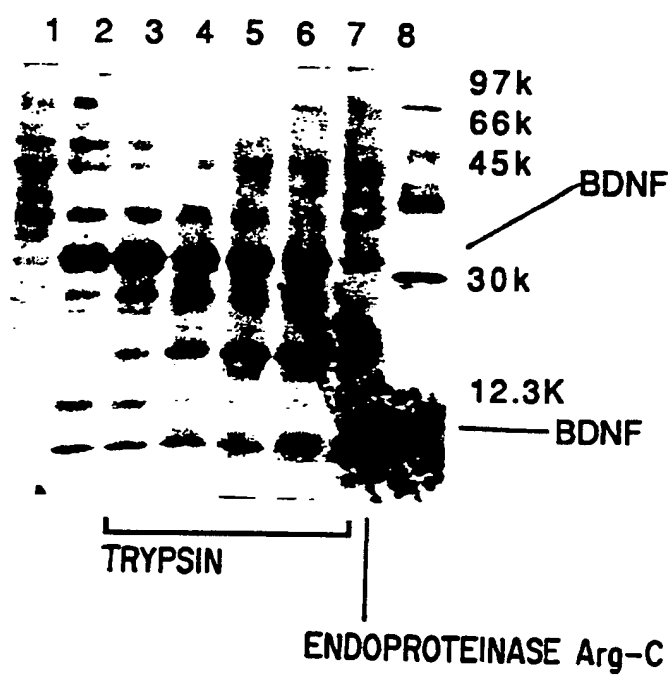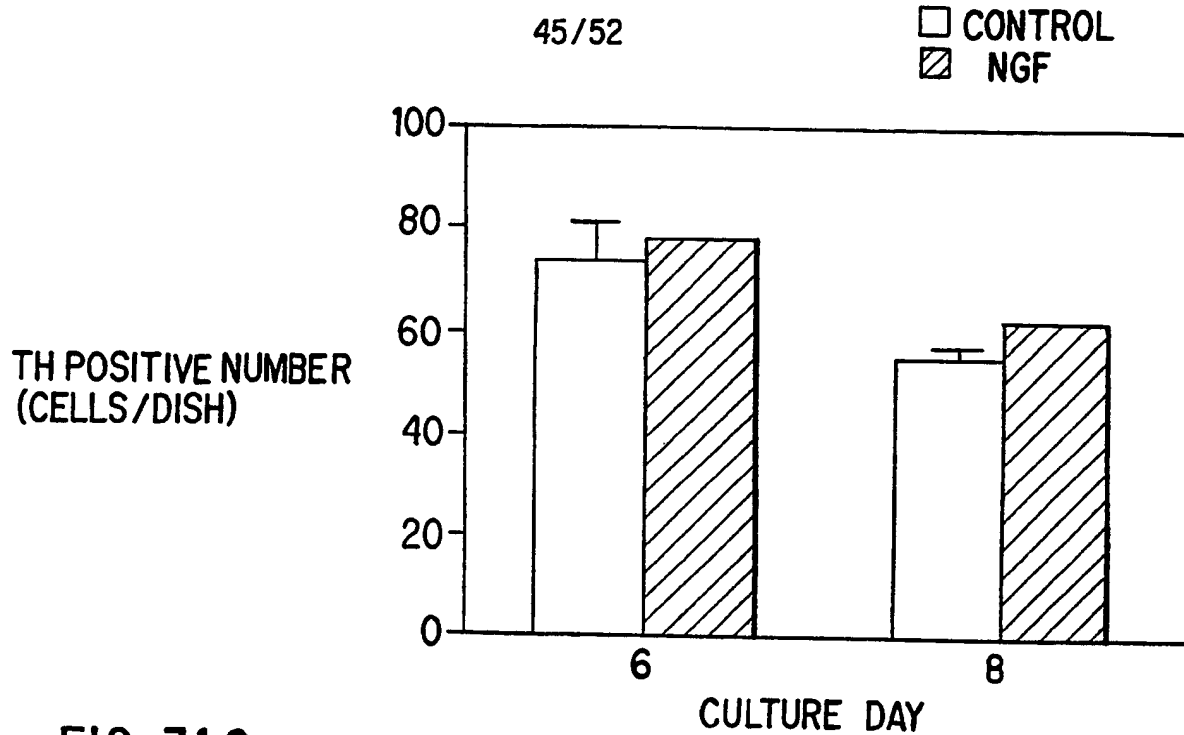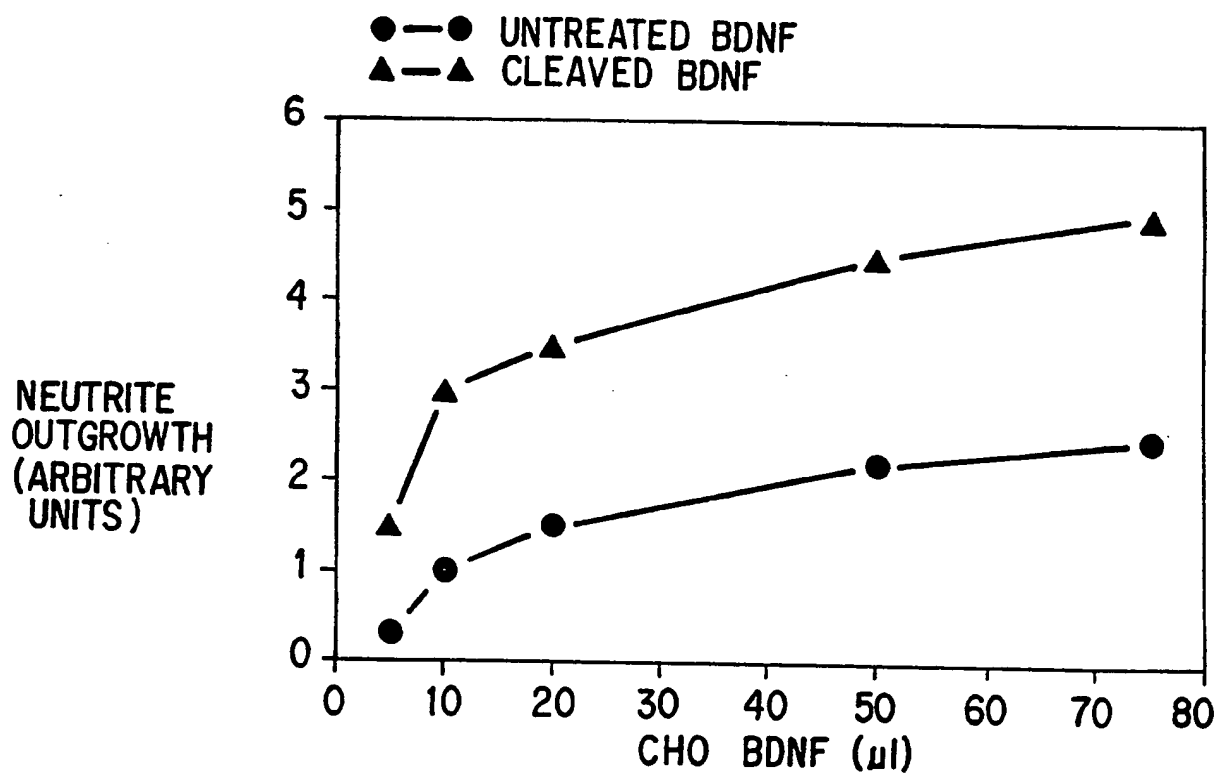
FIG. 31

FIG. 32A

FIG. 32B

FIG. 32C

FIG. 33A

FIG.33B

FIG.33C

□ CONTROL
▨ BDNF

FIG. 34 A

TH POSITIVE
CELL NUMBER
(CELLS/DISH)

CULTURE DAY

FIG. 34 B

TH POSITIVE
CELL NUMBER
(CELLS/DISH)

BDNF (ng/ml)
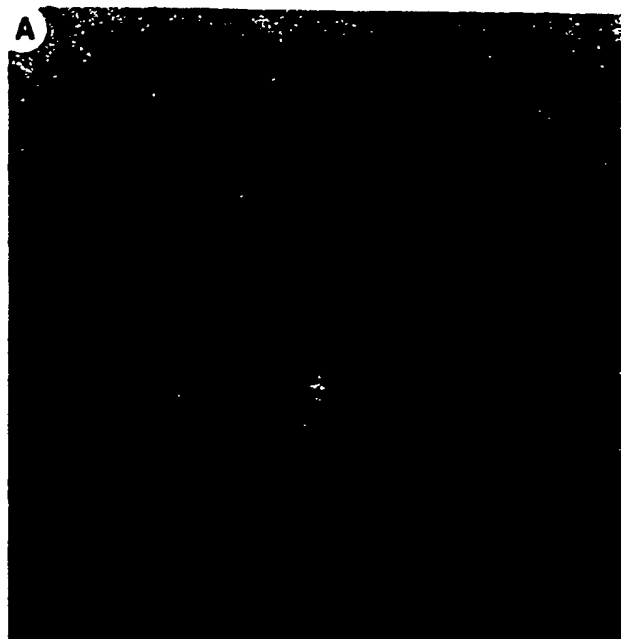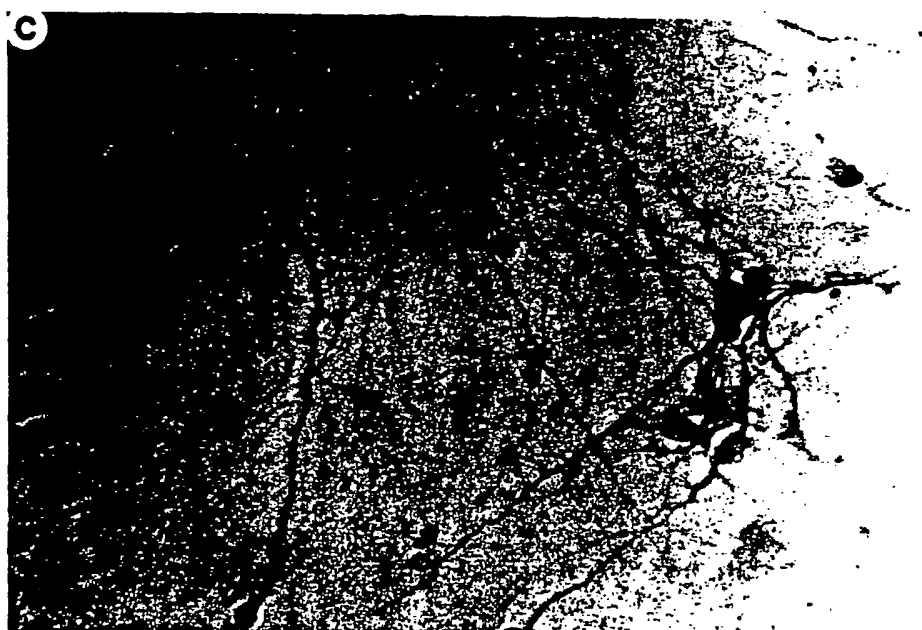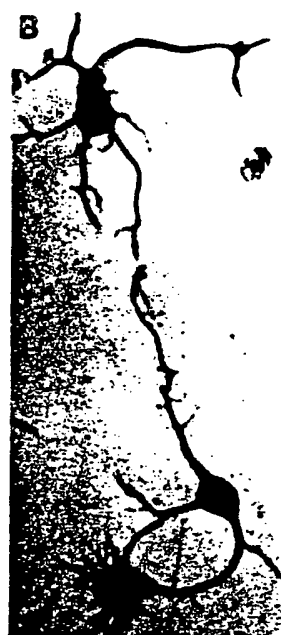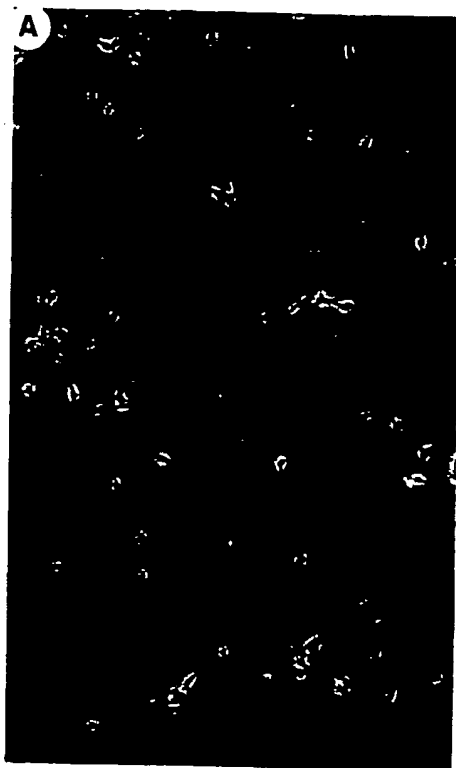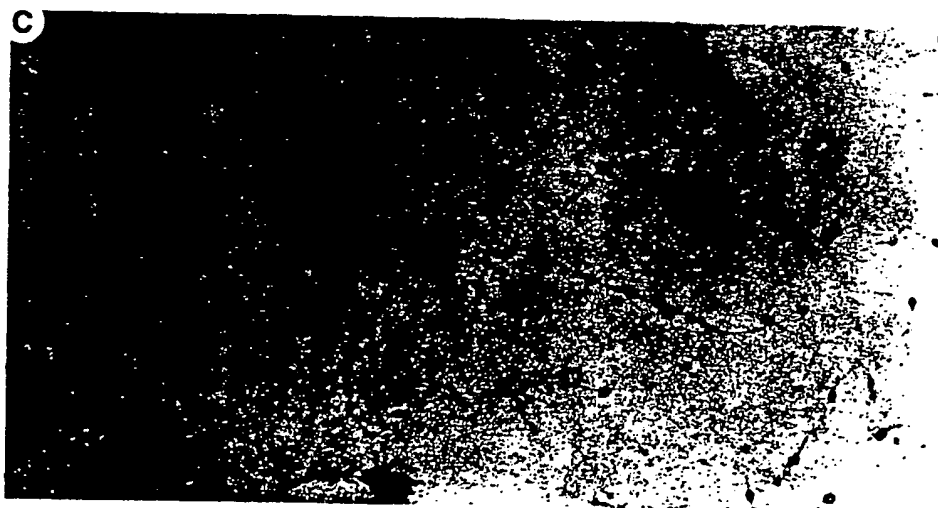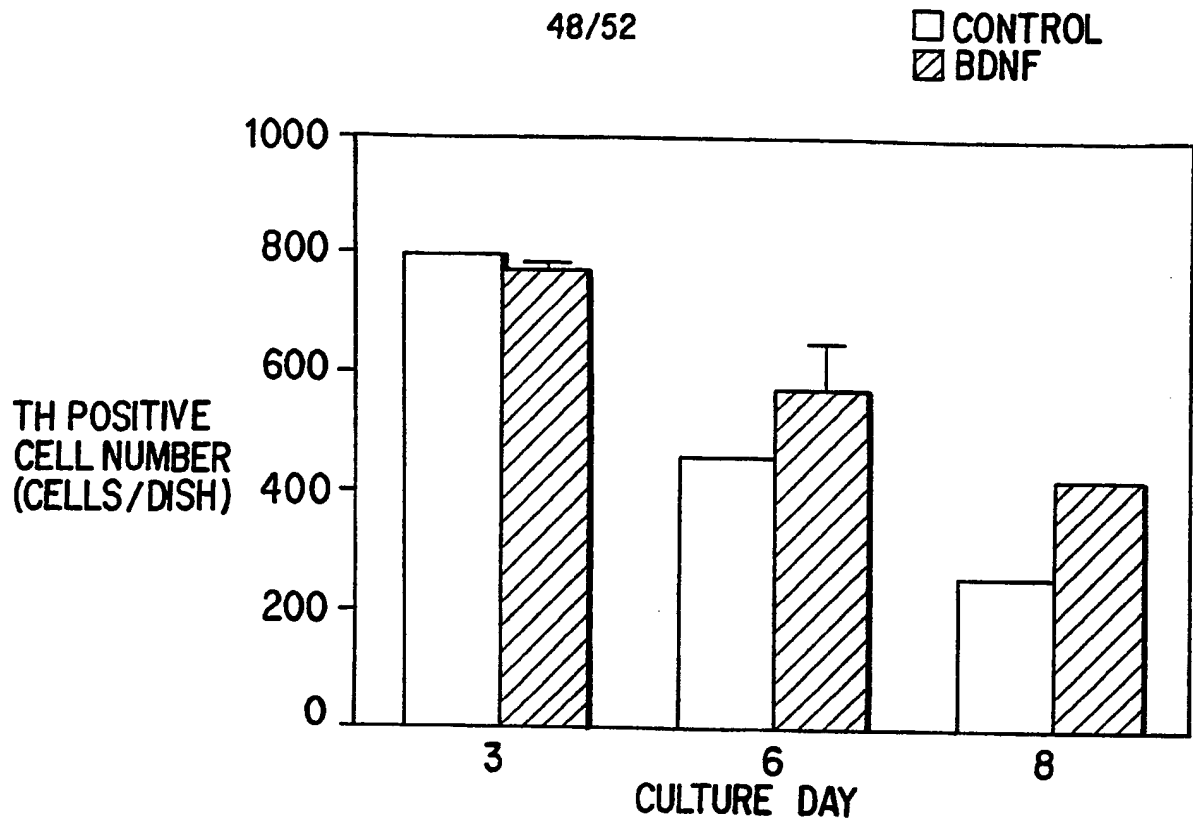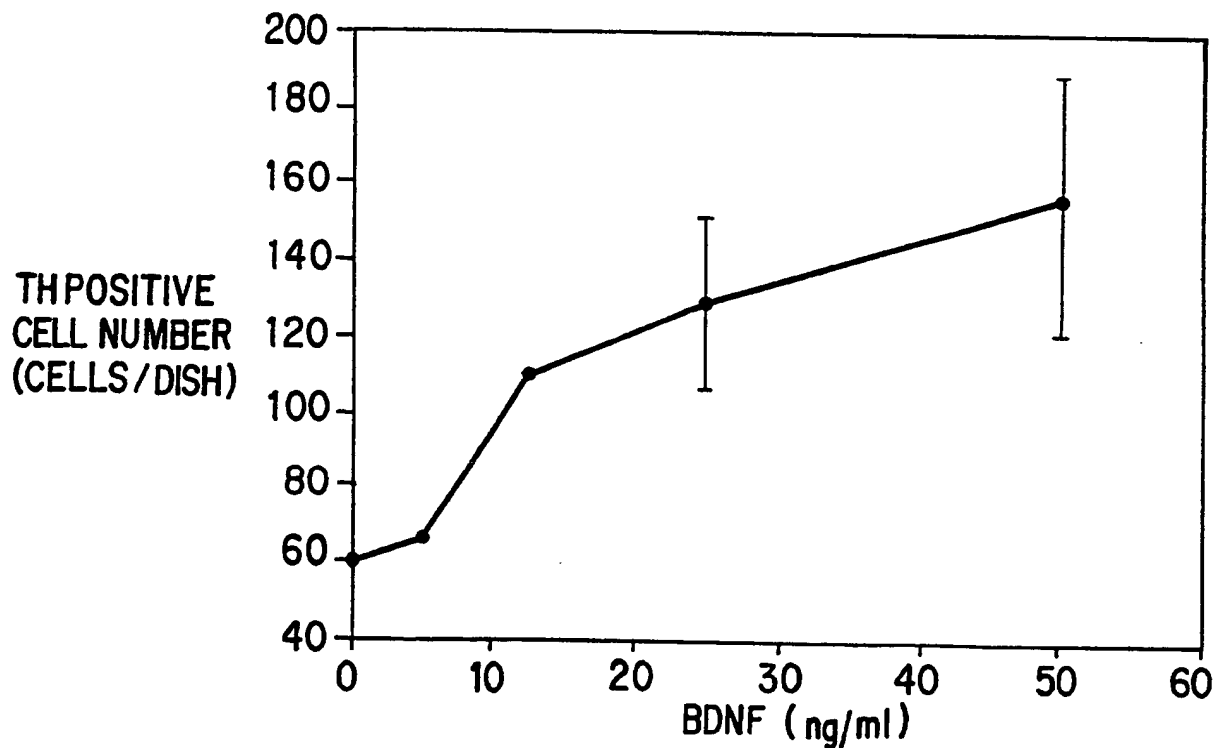
TH POSITIVE CELL
NUMBER (CELLS/DISH)
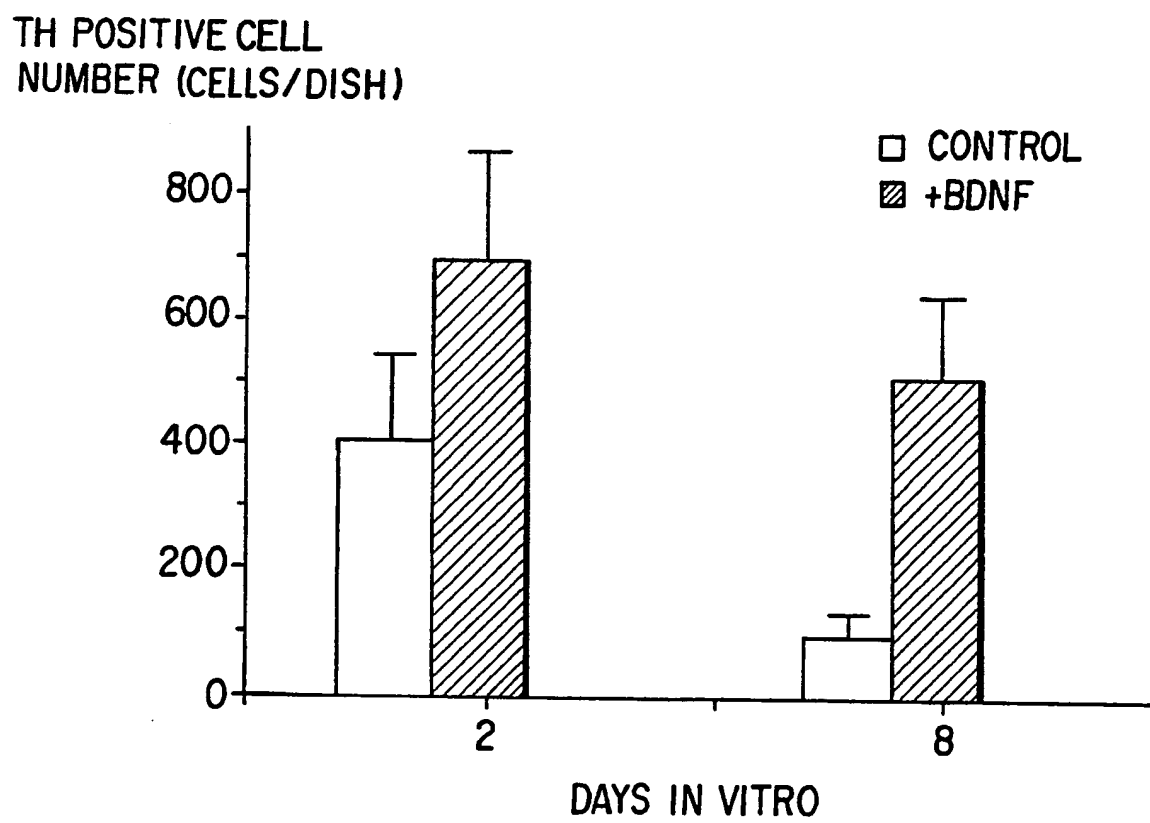
FIG. 35
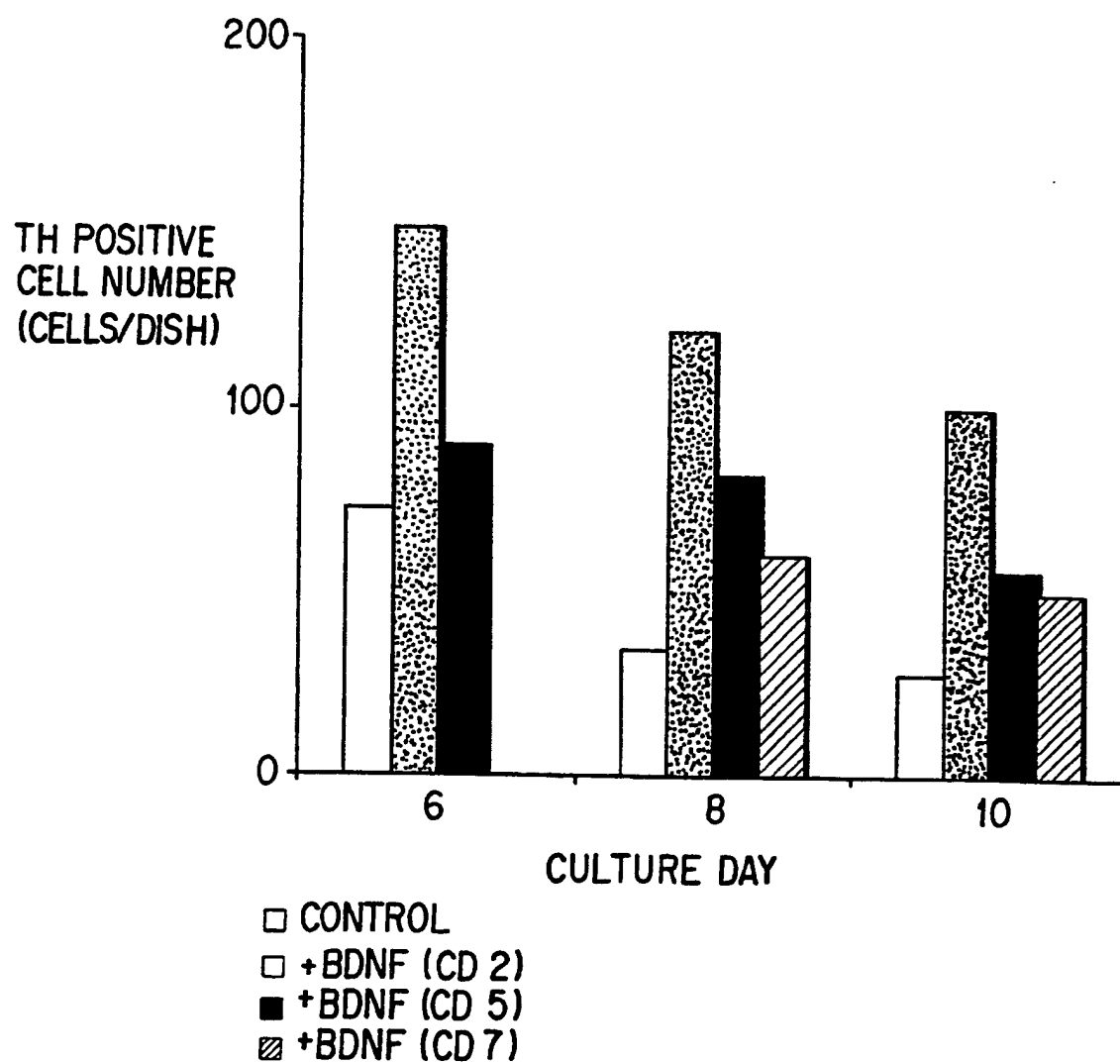
FIG. 36

SPECIFIC GABA UPTAKE
(CPM/10min/well)
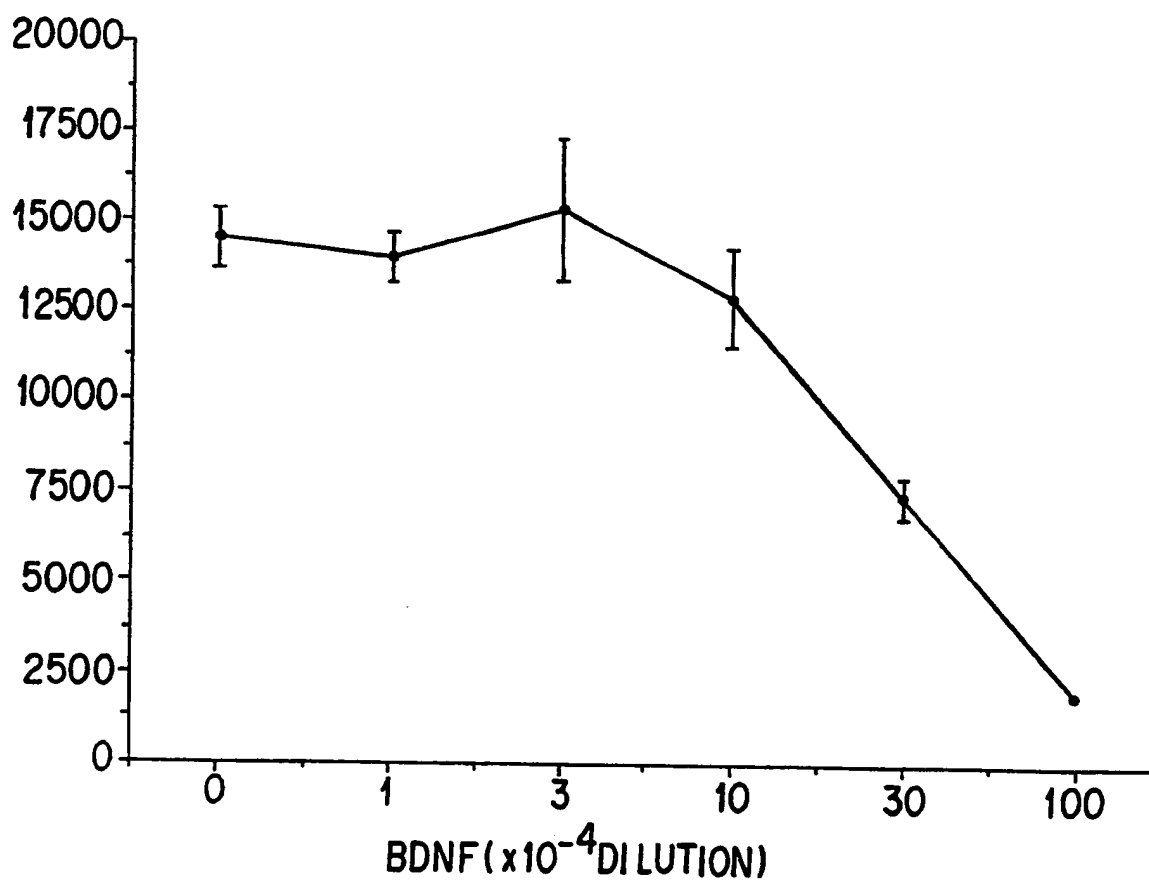
FIG. 37

THE POSITIVE CELL
NUMBER (% OF NON-
MPP+ TREATED)
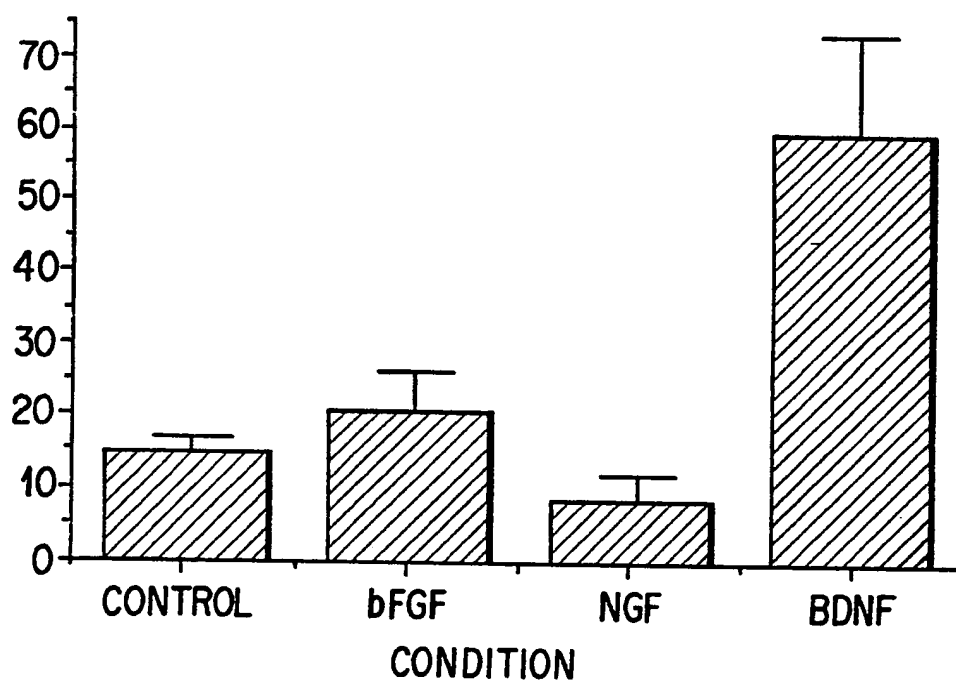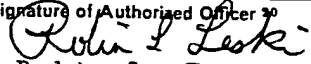
FIG. 38

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/04915

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12P 21/00; C12N 15/00; C12Q 1/00; C07H 15/12
U.S.CL: 435/69.1,69.4,172.3; 530/350,387; 514/12

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System	Classification Symbols
U.S.	435/69.1,69.4,69.6,69.7,320,172.3 536/27; 514/12; 530/387,350; 935/13

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁵

COMPUTER SEARCH INTELLIGENTICS (DNA, PROTEIN SEQUENCE), NGF, BDNF, NEUROTROPHIA, NEUROTROPHIC, PCR, ALZHEIMER & NGF, CONSENSUS PRIMER, SEQUENCE SPECIFIC PRIMER

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	Gene, Volume 70, issued 1988, G. Hu et al.: "Expression of the cDNA for mouse β-nerve growth factor protein in _Escherichia coli_" pp. 57-65. See the entire document.	1-148
Y	Chemical Abstracts, Volume 102, issued 1985, S. H. Appel et al.; "Neuropeptides and Alzheimer's disease: potential role of neurotrophic factors" see the abstract p. 401, Abstract No. 43871b.	66-114,135-141, 148
Y	Chemical Abstracts, Volume 102, issued 1985, S. H. Appel et al.; "New approaches to Alzheimer's disease: neurotrophic factors", see the abstract p. 416, Abstract No. 147006.	66-114,135-141, 148

* Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²
10 November 1990	**11 JAN 1991**

International Searching Authority ¹	Signature of Authorized Officer ²⁰
ISA/US	Robin L. Teskin

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y,P	Chemical Abstracts, Vol. 111, issued November 1989, Y. Uchida et al.; "Neurotrophic factors in Alzheimer's disease brain", see the abstract, p. 203, Abstract No. 229,882X.	66-114,135-141, 148
X,P	Nature, Volume 341, issued 14 September 1989, J. Leibrock et al., "Molecular cloning and expression of brain-derived neurotrophic factor" pp. 149-152, see the entire article.	1-7,10-14,16-37, 39-48,54-67,115-119, 133-134
Y,P		8-9,15,38,49-53, 68-114,120-132, 135-148

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers ...., because they relate to subject matter [1] not required to be searched by this Authority, namely:

2.☐ Claim numbers..........., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3.☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No[18]
X,P Y,P	Nature, Volume 344, issued 22 March 1990, A. Hohn et al. "Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family" pp. 334-341, see the entire article.	1-7,10-14,16-37, 39-48,54-67,115-119, 133-134 8-9,15,38,49-53, 68-114,120-132, 135-148
Y,P	Science, Vol. 246, issued 24 November 1989, L. H. Lin et al., "Purification Cloning, and Expression of Ciliary Neurotrophic Factor (CNTF)" pp. 1023-1025, see the entire article.	1-148
Y	Molecular and Cellular Biology, Vol. 8, No. 1, issued January 1988, F. Hallböök et al., "Production and Characterization of biologically active recombinant beta nerve growth factor" pp. 452-456 see the entire article.	1-148
Y	Nature, Volume 331, issued 04 February 1988, H. Erlich et al.; "Specific DNA amplification", pp. 461-462, see the entire article.	115-119

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Form PCT/ISA/210 (extra sheet) (May 1986)